PHARMACOGENETICS OF VIGABATRIN AND CLOBAZAM

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Author's declaration

I, Prathibha Elizabeth Ann Jose, declare that the research presented in this thesis was carried out by myself in the Department of Pharmacology at University of Liverpool. It is an original piece of work. The views expressed are solely of the author.

Prathibha Elizabeth Ann Jose

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Abbreviations

ABAT	4-aminobutyrate aminotransferase gene
ACTH	adrenocorticotrophic hormone.
ACN	acetonitrile
AEDs	antiepileptic drugs
AET	2-aminoethylisothiouronium bromide hydrobromide
ANOVA	analysis of variance
bp	base pairs
BRCA 1	breast cancer 1, early onset
BRCA 2	breast cancer 2, early onset
BSA	bovine serum albumin
BZDs	benzodiazepines
C/D	concentration divided by dose ratio
Cl^-	chloride
Ca+	calcium
CBZ	carbamazepine
CNS	central nervous system
CLB	clobazam
CLOPS	clobazam prospective cohort study
CNV	copy number variation
CYP 450	cytochrome P450
DNA	deoxyribonucleic acid
EEG	electroencephalograph
EM	extensive metaboliser
ESE	exon splicing enhancers
ESM	ethosuximide
FDA	Food and Drug Administration
FBM	felbamate
FRET	fluorescence resonance energy transfer
GABA	gamma-aminobutyric acid
GABA-T	GABA transaminase enzyme
GBP	gabapentin

GC-MS	gas chromatography–mass spectrometry
G-6PD	glucose-6-phophate dehydrogenase
GWAS	genome wide association study
H-W	Hardy-Weinberg
HER2/NEU	human epidermal growth factor receptor 2
HQC	high quality control
InDel	insertion/deletion
IS	internal standard
K^+	potassium
LEV	levetiracetam
LC-MS	liquid chromatography-mass spectrometry
LQC	low quality control
LTA	lymphotoxin-α
LTG	lamotrigine
MQC	medium quality control
MAF	minor allele frequency
NADH	dihydronicotinamide adenine dinucleotide-coenzyme Q reductase
NAD^+	dihydronicotinamide adenine dinucleotide
Na^+	sodium
NC	negative control
NCBI	National Centre for Biotechnology Information
NDCB	N-desmethylclobazam
NICE	National Institute for Health and Clinical Excellence
nm/mg/ml	nanometre/milligram/ml
OCP	oral contraceptive pill
OXC	oxcarbazepine
PB	phenobarbital
PCR	polymerase chain reaction
PAR	peak area ratio
PHT	phenytoin
PM	poor metabolizer
PPI	proton pump inhibitor

QC	quality control
RBC	red blood cell
RMP	resting memberane potential
RNA	ribonucleic acid
sec	seconds
SNP	single nucleotide polymorphism
STR	short tandem repeat
SEM	standard error of the mean
SV2A	synaptic vesicle protein 2A
TCA	tricyclic antidepressant
TDM	therapeutic drug monitoring
TGB	tiagabine
TPM	topiramate
UM	ultra rapid metabolizer
VGB	vigabatrin
VKORC1	vitamin K epoxide reductase complex subunit 1
VNTR	varying number of tandem repeat
VPA	valproic acid
VVFD	vigabatrin induced visual field defect
VFD	visual field defect
ZNS	zonisamide
5HT3 receptor	5-hydroxytryptamine receptor

Publications and communications

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Prathibha Jose, Ana Alfirevic, Tim P Green, Munir Pirmohamed. GABA-transaminase – characterisation of phenotype-genotype relationship. Manuscript in preparation.

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Abstract

Both vigabatrin (VGB) and clobazam (CLB) are effective antiepileptic drugs (AEDs) that act on the GABA system. There is significant variation in the efficacy and toxicity of many AEDs including VGB and CLB. The aim of the thesis was to investigate the mechanisms responsible for the variation in response to VGB and CLB.

VGB is used as add-on therapy and is particularly important in the treatment of infantile spasms. However it causes visual field defect (VFD) in around 30-40% of patients, the aetiology of which is not yet fully understood. Genetic variants in the drug target could be important. VGB inhibits the GABA transaminase (GABA-T) enzyme which catabolises GABA, the primary inhibitory neurotransmitter in the central nervous system (CNS). We investigated the effect of genetic variants on GABA-T activity in healthy volunteers. A platelet GABA-T activity assay was standardised. The nonsynonymous single nucleotide polymorphism (SNP), rs1731017, reduced GABA-T activity in healthy volunteers, although there is no evidence to suggest that it is associated with VFD in patients. GABA-T activity, measured in platelets of patients with VGB-induced VFD was 2-fold lower than in patients who had been on VGB but had not developed VFD. The molecular basis of this is unknown, but needs further investigation.

CLB is efficacious in many types of epilepsies, but is also associated with tolerance. Drowsiness, dizziness and weight gain are its major adverse effects. There is a large variation in dose, plasma concentrations and the occurrence of tolerance among patients. Genetic variants affecting the pharmacokinetic pathways could influence the metabolism, efficacy and toxicity of CLB. CYP2C19 is the main enzyme

involved in the metabolism of CLB and its major metabolite is N-desmethylclobazam (NDCB). Therefore we investigated the influence of *CYP2C19* genetic variants on the metabolism, efficacy and toxicity of CLB and NDCB. First we developed a high pressure liquid chromatography (HPLC) assay to estimate the concentration of CLB and NDCB. This assay was reproducible with high specificity and sensitivity. CYP2C19*2 (rs4244285) homozygous minor allele patients had 5.7- and 4.7-fold higher NDCB concentration than homozygous major allele and heterozygous patients, respectively. There was no significant difference between the homozygous major and heterozygous groups. The number of patients who achieved more than 50 percent seizure reduction (p=0.05) and seizure freedom (p=0.014) was significantly higher in those with homozygous minor alleles when compared to patients with heterozygous and homozygous major alleles of CYP2C19*2. CYP2C19*2 was a covariate for NDCB/CLB ratio and efficacy. Patients homozygous for CYP2C19*17 (rs12248560) were on a significantly higher dose of CLB than patients lacking this allele (p=0.016). rs11568732 offered protection against the development of dizziness (p=0.04).

In summary, in this thesis, I have focused on 2 drugs acting on the GABA system. The identification of a phenotypic difference in GABA-T activity between patients with and without vigabatrin induced visual field defect (VVFD) is a significant finding which needs further investigation. CYP2C19 plays an important role in the metabolism of CLB. The data show that polymorphisms in the *CYP2C19* gene affect the pharmacokinetics of CLB, and importantly may also influence efficacy and toxicity. The data presented in this thesis show that further investigation to individualise therapy with VGB and CLB, based on both clinical and genetic factors, is a research area that merits further attention.

Chapter 1

Introduction

1.1. Epilepsy

Epilepsy is a common chronic neurological disorder characterized by spontaneous recurrence of unprovoked seizures. Epilepsy affects people of all ages. The disorder is not fully understood; therefore classification of seizure types and syndromes is ongoing and often not possible in many patients. Many factors affect the classification of epilepsy, complicating the assessment and treatment of individuals. These include concomitant medical conditions, learning disabilities, neurological deficits, psychological, psychiatric problems and progressive conditions (Forsgren, 2004).

1.1.1. Classification of epilepsy

The most accepted classification of epilepsy is the International League Against Epilepsy classification which was published in 1989 (Dodson, 2004). However, recommendations to alter this classification have been made (Berg, et al., 2010; Shinnar, 2010). The new information that has accrued through modern neuroimaging, genomic technologies, and advances in molecular biology has now been incorporated in this classification. There is a departure from the previous classification which was mainly based on seizure patterns, development (focal or generalised), associated features (loss of consciousness) and epilepsy syndromes. The present classification has tried to look at the seizure presentation, seizure type, epileptic syndrome, aetiology and associated mental retardation. This helps the clinician to make a better diagnosis and administer therapeutic strategies to patients even if they depart from the prototype. The complete classification is reproduced in appendix 1 (Commission

on Classification and Terminology of the International League Against Epilepsy, 2010).

1.1.2. Epidemiology of epilepsy

The incidence of epilepsy in developed countries is about 40-70 per 100,000 people per year. Recent studies have suggested that the incidence of epilepsy in developing countries is approximately twice that in industrialized countries. Poor sanitation, inadequacies in the health system and higher risk of brain infections could be contributing to the higher incidence found in the former. 90% of epilepsy cases worldwide are from the developing countries. The prevalence of epilepsy in developed countries is approximately 4-10 per 1,000 people, and that in developing countries is about 6 to 10 per 1,000 with the cumulative lifetime incidence being about 3%. Around 50 million people in the world have epilepsy (Forsgren, 2004).

1.1.3. Aetiology of epilepsy

The aetiology of epilepsy in a large proportion of patients is not known (50-70%). Other causes include head trauma, central nervous system infections and tumors. For the younger population, perinatal complications, congenital, developmental and genetic conditions are the most common causes of epilepsy. Cerebrovascular disease is the most common risk factor in the elderly. A family history of epilepsy seems to increase the propensity to develop epilepsy (Forsgren, 2004).

1.1.4. Mechanisms of seizure generation

Brain injury due to the above causes is important in the aetiology of epilepsy. Recent evidence shows that an epileptic region of the brain consists of multiple small distributed hyperexcitable networks. Abnormal discharges which are not detectable

clinically occur in this network. Clinical seizures occur when micro seizures enlarge and affect the surrounding normal regions of the brain (Dichter, 2009). Neurochemically, an imbalance between excitatory and inhibitory neurotransmission processes has been proposed (Figure 1.1). The resting membrane potential (RMP) of a neuron is -70mV. Any of the factors which increase this membrane potential can predispose to seizure production. Sodium influx, calcium currents and paroxysmal depolarization raises the membrane potential which could lead to seizures. Potassium efflux, chloride influx and low pH tend to lower the RMP, thereby inhibiting neurons and preventing seizures. Seizures occur when the factors which predispose to seizures predominate over those which inhibit seizures, as illustrated in Figure 1.1.

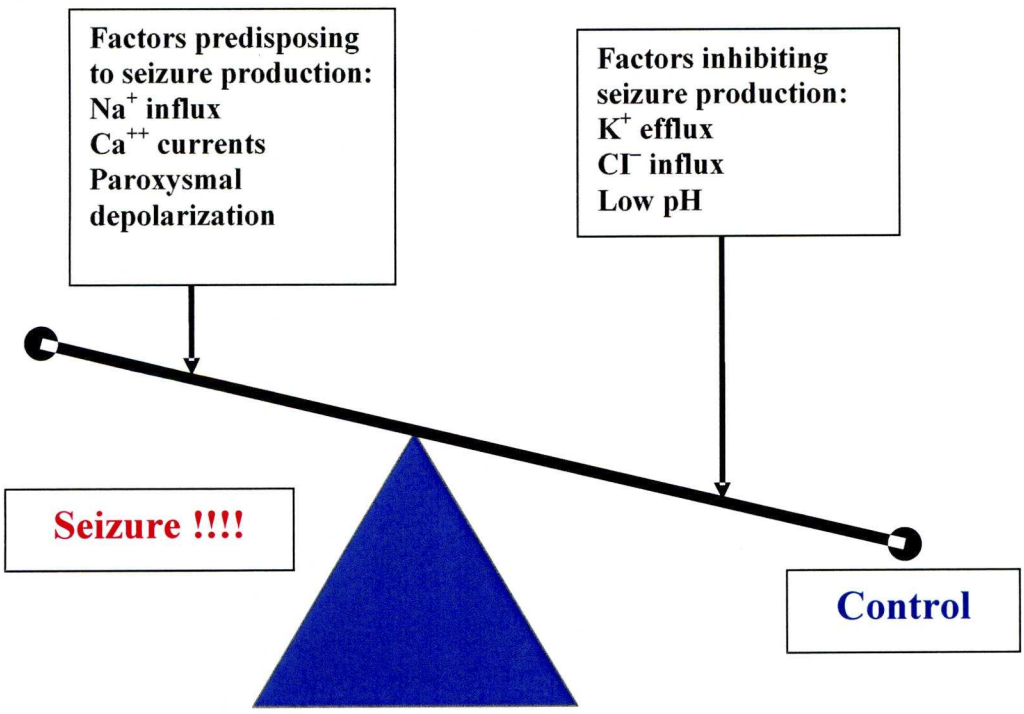


Figure 1.1. Schematic diagram representing the factors predisposing to, and factors inhibiting seizure production. An imbalance can lead to seizure production. Na^+ - sodium; Ca^{++} - calcium; K^+ - potassium; Cl^- - chloride

Neurotransmission is facilitated by action potentials. An action potential is caused by an exchange of ions across the neuronal membrane. During an action potential, due to a stimulus, sodium channels open leading to an influx of sodium ions as shown in Figure 1.2. This makes the neurons more positive and depolarized (from -70 mV to +30 mV). The potassium channels then open causing an efflux of potassium ions, leading to repolarization. The sodium channels close at this time restoring the resting membrane potential as shown in the Figure 1.2. This is an excitatory postsynaptic potential. Glutamate and acetylcholine facilitate this transmission. There are inhibitory postsynaptic potentials in which negative ions flow into the cells or positive ions flow out of the cells, inhibiting the propagation of nerve impulses. Gamma aminobutyric acid (GABA) is an inhibitory neurotransmitter which reduces depolarization of neurons. In epilepsy, there is repetitive firing of neurons, with repetitive stimulation of inhibitory synapses. However the inhibitory events are not successful in controlling the excitatory activity resulting in seizures. Calcium channels are involved in neurotransmission in the thalomocortical region which has been implicated in absence seizures.

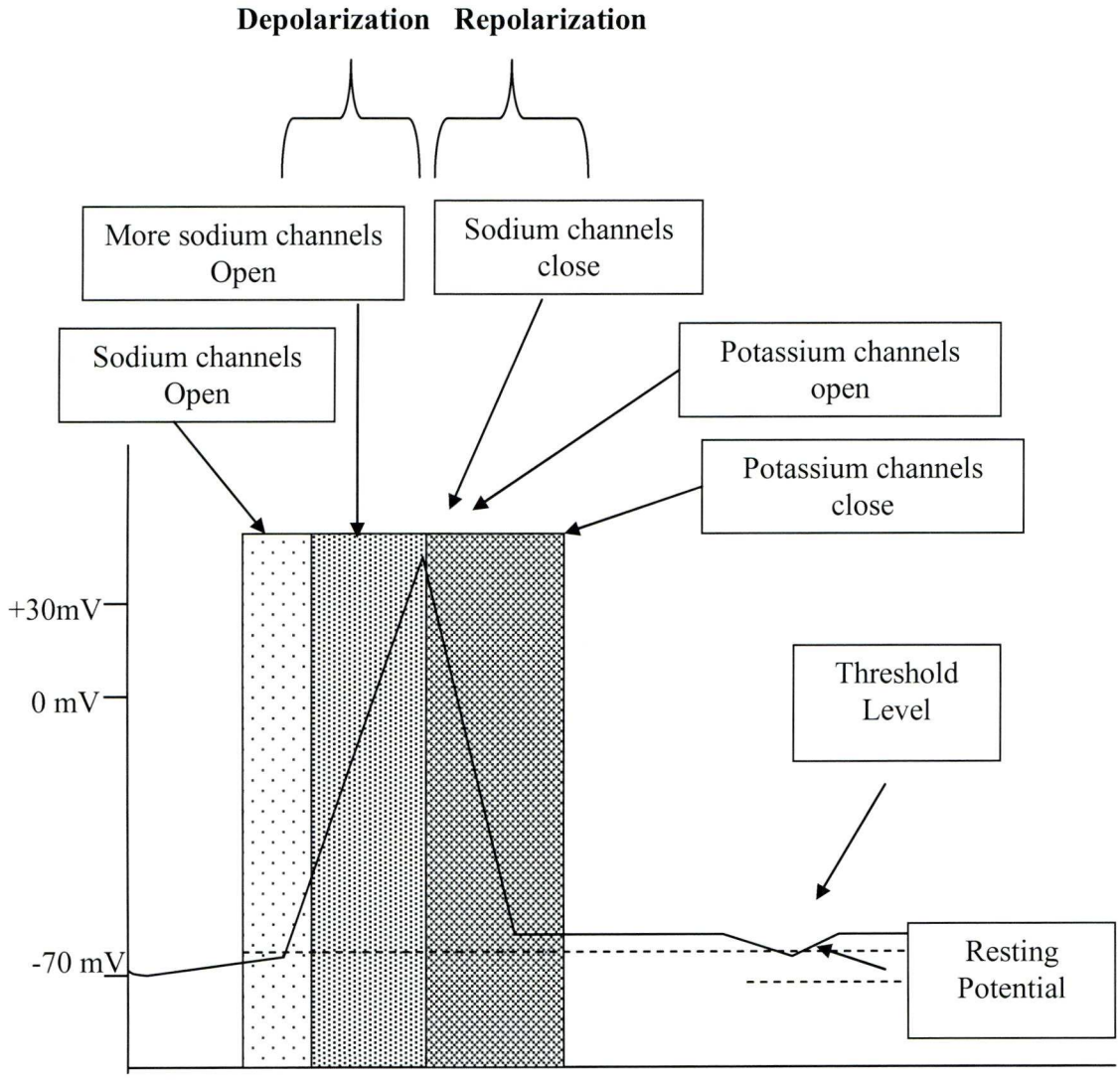


Figure 1.2. A schematic representation of an action potential

1.1.5. Treatment of epilepsy

The treatment of epilepsy depends on the epilepsy type (Table 1.1), epilepsy syndrome (Table 1.2), age of the patient and whether the patient is pregnant or not (http://www.nice.org.uk/CG020NICEguideline). AEDs are the mainstay of treatment of epilepsy. Around 70% of the patients respond to the currently available treatment. Other treatment options include ketogenic diet and surgery.

Usually drug treatment is started with a single drug at low dose and slowly increased at 1-2 week intervals. This enables clinicians to evaluate the clinical progress after each change in dose/ drug and also to avoid adverse drug effects. If patients still have seizures after achieving the full dose of the first drug, the second drug is introduced slowly. Information sheets about drugs are given to patients. Blood levels of drugs are used to monitor compliance, possible adverse effects and reasons for incomplete seizure control (http://www.nice.org.uk/CG020NICEguideline).

Table 1.1. Drug options by seizure type

Seizure type	First-line drugs	Second-line drugs	Other drugs that may be considered	Drugs to be avoided (may worsen seizures)
Generalised tonic–clonic	Carbamazepine[a] Lamotrigine[b] Sodium valproate Topiramate [a,b]	Clobazam Levetiracetam Oxcarbazepine[a]	Acetazolamide Clonazepam Phenobarbital[a] Phenytoin[a] Primidone [a,c]	Tiagabine Vigabatrin
Absence	Ethosuximide Lamotrigine[b] Sodium valproate	Clobazam Clonazepam Topiramate[a]		
Myoclonic	Sodium valproate (Topiramate [a,d])	Clobazam Clonazepam Lamotrigine Levetiracetam Piracetam Topiramate[a]	Carbamazepine[a] Gabapentin Oxcarbazepine[a] Tiagabine Vigabatrin	
Tonic	Lamotrigine[b] Sodium valproate	Clobazam Clonazepam Levetiracetam Topiramate[a]	Acetazolamide Phenobarbital[a] Phenytoin[a] Primidone[a,c]	Carbamazepine[a] Oxcarbazepine[a]
Atonic	Lamotrigine[b] Sodium valproate	Clobazam Clonazepam Levetiracetam Topiramate[a]	Acetazolamide Phenobarbital[a] Primidone [a,c]	Carbamazepine[a] Oxcarbazepine[a] Phenytoin[a]
Focal with/ without secondary generalisation	Carbamazepine[a] Lamotrigine[b] Oxcarbazepine[a,b] Sodium valproate Topiramate [a,b]	Clobazam Gabapentin Levetiracetam Phenytoin[a] Tiagabine	Acetazolamide Clonazepam Phenobarbital [a] Primidone [a,c]	

a. Hepatic enzyme-inducing AED.

b. Should be used as a first choice when the older drugs do not stop seizures or cause adverse effects

c. Should rarely be initiated – if a barbiturate is required, phenobarbital is preferred.

d. In children, for severe myoclonic epilepsy of infancy (see Table 2).

Adopted from NICE guidelines (http://www.nice.org.uk/CG020NICEguideline)

Table1. 2. Drug options by epilepsy syndrome

Epilepsy syndrome	**First-line drugs**	**Second-line drugs**	**Other drugs**	**Drugs to be avoided (may worsen seizures)**
Childhood absence epilepsy	Ethosuximide Lamotrigine [b] Sodium valproate	Levetiracetam Topiramate[a]		Carbamazepine[a] , Phenytoin[a], Oxcarbazepine[a], Tiagabine Vigabatrin
Juvenile absence epilepsy	Lamotrigine[b] Sodium valproate	Levetiracetam Topiramate[a]		Carbamazepine[a] , Phenytoin[a], Oxcarbazepine[a], Tiagabine Vigabatrin
Juvenile myoclonic epilepsy	Lamotrigine[b] Sodium valproate	Clobazam Clonazepam Levetiracetam Topiramate [a]	Acetazolamide	Carbamazepine[a] , Phenytoin[a], Oxcarbazepine[a], Tiagabine Vigabatrin
Generalised tonic–clonic seizures	Carbamazepine[a] Lamotrigine[b] Sodium valproate Topiramate [a,b]	Levetiracetam	Acetazolamide Clobazam Clonazepam Oxcarbazepine[a] Phenobarbital[a] Phenytoin[a] Primidone [a,c]	Tiagabine Vigabatrin
Focal epilepsies: cryptogenic, symptomatic	Carbamazepine[a] Lamotrigine[b] Oxcarbazepine [a,b] Sodium valproate Topiramate [a,b]	Clobazam Gabapentin Levetiracetam Phenytoin[a] Tiagabine	Acetazolamide Clonazepam Phenobarbital[a] Primidone [a,c]	
Infantile spasms	Steroids[c] Vigabatrin[b]	Clobazam Clonazepam Sodium valproate Topiramate[a]	Nitrazepam	Carbamazepine[a] Oxcarbazepine[a]

Benign epilepsy with centrotemporal spikes	Carbamazepine[a] Lamotrigine[b] Oxcarbazepine [a,b] Sodium valproate	Levetiracetam Topiramate[a]	Sulthiame[e]	
Benign epilepsy with paroxysms	Carbamazepine[a] Lamotrigine[b] Oxcarbazepine [a,b] Sodium valproate	Levetiracetam Topiramate[a]		
Severe myoclonic epilepsy of infancy	Clobazam Clonazepam Sodium valproate Topiramate [a,b]	Levetiracetam Stiripentole	Phenobarbital[a]	Carbamazepine[a] Lamotrigine Oxcarbazepine[a] Vigabatrin
Continuous spike wave of slow sleep	Clobazam Clonazepam Ethosuximide Lamotrigine[b] Sodium valproate Steroids[d]	Levetiracetam Topiramate[a]		Carbamazepine[a] Oxcarbazepine[a] Vigabatrin
Lennox–Gastaut syndrome	Lamotrigine[b] Sodium valproate Topiramate [a,b]	Clobazam Clonazepam Ethosuximide Levetiracetam	Felbamate[e]	Carbamazepine[a] Oxcarbazepine[a]
Landau–Kleffner syndrome	Lamotrigine[b], Steroids[d] Sodium valproate	Levetiracetam Topiramate[a]	Sulthiame [e]	Carbamazepine[a] Oxcarbazepine[a]

a). Hepatic enzyme-inducing AED. b). Should be used as a first choice when the older drugs do not stop seizures or cause adverse effects. c). should rarely be initiated if a barbiturate is required, phenobarbital is preferred. d) Steroids: prednisolone or ACTH (adrenocorticotrophic hormone). e). Not licensed in the UK, but available by importation. Adopted from NICE guidelines (http://www.nice.org.uk/CG020NICEguideline).

1.1.6. Antiepileptic drugs

There is an armamentarium of AEDs available on the market to treat epilepsy patients. AEDs can be grouped according to their major mechanisms of action. Most of the AEDs act on a combination of ion channels but may also have other unknown mechanisms of action (Meldrum, 1996).

Mechanism of action of AEDs:

In a nutshell:

1. Some AEDs stabilize the inactive configuration of the **sodium (Na^+) channel**, preventing high-frequency neuronal firing, e.g., phenytoin, carbamazepine, lamotrigine, oxcarbazepine.
2. **Low-voltage calcium ($Ca2^{+)}$ currents (T-type)** are responsible for the rhythmic thalamocortical spike and wave patterns of generalized absence seizures. Some AEDs block these channels, inhibiting the underlying slow depolarizations necessary to generate spike-wave bursts, e.g., ethosuximide, valproic acid.
3. The **GABA-A receptor** mediates chloride (Cl^-) influx, leading to hyperpolarization of the cell and inhibition. AEDs may act to enhance Cl^- influx, e.g., phenobarbital, benzodiazepines.
4. **Glutamate**, the main excitatory neurotransmitter in the CNS, binds to multiple receptor sites that differ in activation and inactivation time courses, desensitization kinetics, conductance, and ion permeability. AEDs that modify these receptors are antagonistic to glutamate; e.g., felbamate, topiramate.
5. **Carbonic anhydrase inhibition** results in hyperpolarization and an increase in seizure threshold of the cells, e.g., acetazolamide.
6. **Synaptic vesicle protein 2A binding**: SV2A appears to be important for the availability of calcium-dependent neurotransmitter vesicles ready to release their content. Levetiracetam binds to SV2A.

Table 1.3.gives a summary of the proposed mechanisms of AEDs action

Table 1.3. Summary of the proposed mechanisms of antiepileptic drugs (AEDs) action

Established AEDs	Na^+ channels	$Ca2^+$ channels	K^+ channels	Inhibitory transmission	Excitatory transmission
PHT	+++				
CBZ	+++				
ESM		+++			
PB		+		+++	+
BZDs				+++	
VPA	+	+		++	+
New AEDs					
LTG	+++	+			
OXC	+++	+	+		
ZNS	++	++			
VGB				+++	
TGB				+++	
GBP	+	+		++	
FBM	++	++		++	++
TPM	++	++		++	++
LEV		+		+	+

+++, primary action; ++, probable action; +, possible action.
PHT = phenytoin; CBZ = carbamazepine; ESM = ethosuximide; PB= phenobarbital; BZDs = benzodiazepines; VPA= valproic acid; LTG= lamotrigine; OXC = oxcarbazepine; ZNS= zonisamide; VGB = vigabatrin; TGB= tiagabine; GBP= gabapentin; FBM= felbamate; TPM= topiramate; LEV= levetiracetam
Data from Upton, 1994, Schachter, 1995,Macdonald and Kelly, 1995, Meldrum, 1996, Coulter, 1997, White, 1999, adopted from Kwan, et al., 2001.

Many of the AEDs potentiate the effect of GABA. Figure 1.3 shows the AEDs that act on the GABA synapse. GABA is synthesized from glutamic acid by glutamic acid decarboxylase enzymes (GAD1 and GAD2). Valproic acid and gabapentin increase the production of GABA (Patsalos, 2005). GABA is transported to the vesicles by GABA transporters and released into the synapse. Valproic acid is proposed to

augment this (Rowley, et al., 1995). Gabapentin increases the nonvesicular release of GABA (Gotz, et al., 1993).

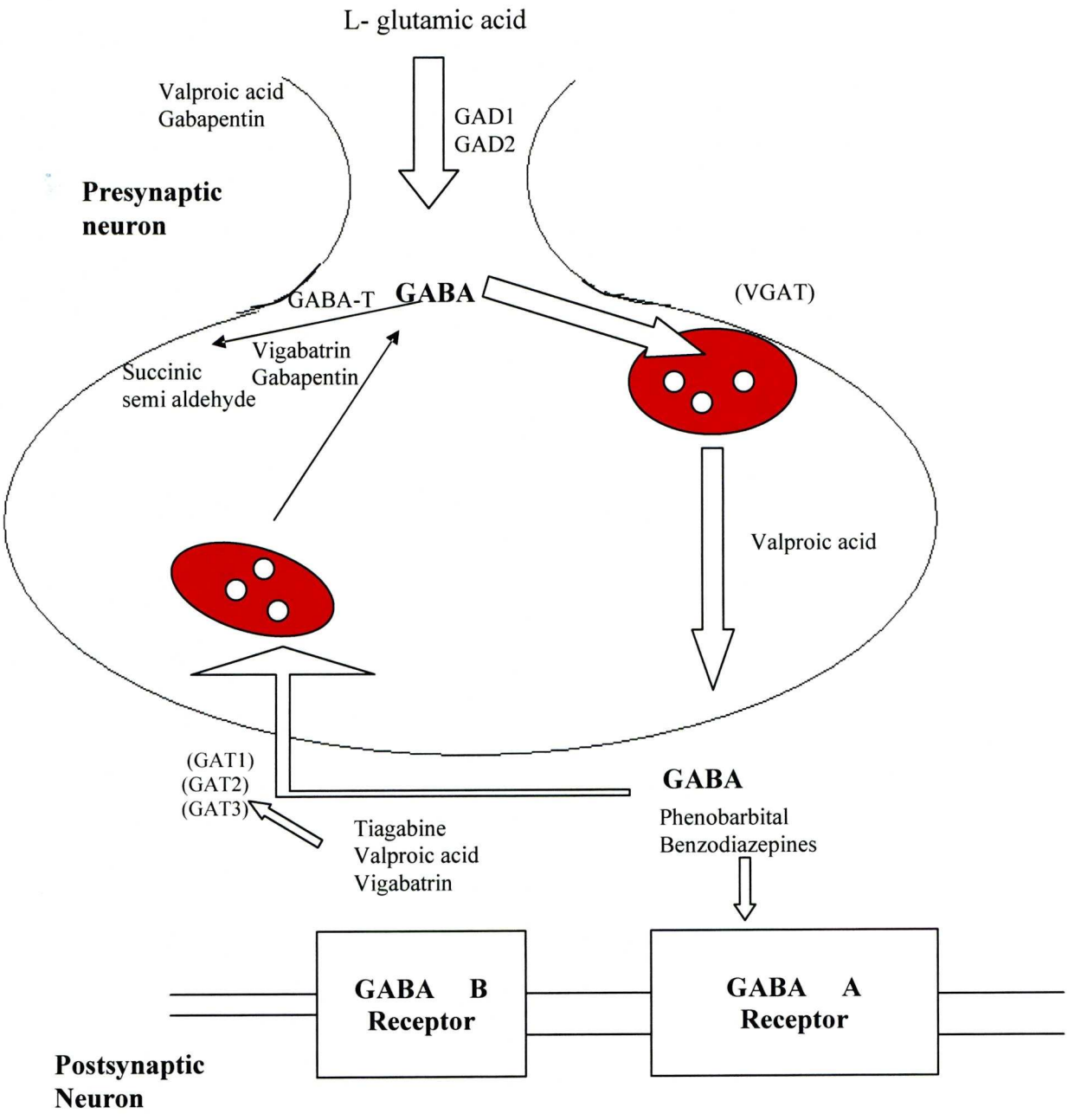


Figure 1.3. Schematic diagram depicting the action of antiepileptic drugs (AEDs) on the GABA synapse. GAT: GABA Transporter, VGAT: Vesicular GABA Transporter, GAD- Glutamic acid decarboxylase, GABA-T- GABA-tr nsaminase enyme

GABA acts on its receptors - GABA A, GABA B and GABA C. GABA A and GABA B receptors are ubiquitous in distribution in the central nervous system (Bowery, et al., 1984). However, GABA C is predominantly present in the retina. Phenobarbital (Macdonald, et al., 1989; Twyman, et al., 1989) and benzodiazepines (Macdonald and Kelly, 1995) act on GABA A receptors and bring about CNS inhibition. From the synapse, GABA reuptake is facilitated by GABA transporters (GAT1, GAT2 and GAT3). Tiagabine inhibits GAT1 (Braestrup, et al., 1990). Valproic acid (Leach, et al., 1996; Sills, et al., 1996) and VGB (Leach, et al., 1996) too are proposed to increase the concentration of GABA by inhibiting GABA uptake. GABA is metabolised by GABA transaminase (GABA-T) enzyme. VGB (Lippert, et al., 1977) and gabapentin (Leach, et al., 1997) inhibit GABA-T and raise the concentration of GABA.

In spite of these drugs, around 30% of epilepsy patients suffer from breakthrough seizures. Clobazam (CLB) (Guberman, et al., 1990; Michael and Marson, 2008) and vigabatrin (VGB) (Bruni, et al., 2000; Guberman and Bruni, 2000) have been used successfully in many of these refractory cases (Loiseau, et al., 1986).

1.2. Clobazam (CLB)

CLB is a 1,5-benzodiazepine that has been available for more than 3 decades. It was first introduced as an anxiolytic. Its antiepileptic property has been known about since 1973 (Barzaghi, et al., 1973; Gastaut and Low, 1979). N-desmethylclobazam (NDCB), its active metabolite, exerts a major influence on its antiepileptic properties and adverse drug reactions (Gastaut and Low, 1979). The onset of action of CLB is fast and it is effective against a broad spectrum of seizure disorders (Munn, et al.,

1988; Canadian Clobazam Cooperative Group, 1991; Michael and Marson, 2008). CLB has been available in Europe since 1973.

1.2.1. Mechanism of action

GABA is the primary inhibitory neurotransmitter in human brain. CLB acts on GABA A postsynaptic receptors present in almost all cortical neurons. This results in allosteric modification of the GABA A receptor and enhances inhibition. Binding of CLB to the benzodiazepine recognition site on the GABA A receptor increases the frequency of GABA A receptor opening without significantly affecting the mean open time or conductance of the channel. CLB can activate the GABA A receptor only in the presence of GABA. CLB acts on the alpha-3 containing GABA A receptors present in reticular neurons and prevents absence seizures. It also increases the number of sodium channels in the inactive state (Meldrum and Chapman, 1986). This could be the reason for termination of generalized epileptic activity on the EEG. CLB also reduces conductance in calcium channels and decreases neurotransmitter release (Meldrum and Chapman, 1986; Sieghart, 1992).

1.2.2. Pharmacokinetics

CLB is absorbed rapidly attaining peak plasma concentration between 1-4 hours. 82-90 % of CLB binds to protein. The free (unbound) CLB is active. It is lipophilic and has a high volume of distribution (Dalby, 2004). The half life of CLB is 11 to 77 hours in healthy subjects and 12 to 13 hours in patients on AEDs which induce the activity of CYP450 (Greenblatt, et al., 1981; Bun, et al., 1990). The half-life of NDCB also follows a similar pattern. The half-life of NDCB is 50 hrs (Bun, et al., 1990). The concentration of NDCB is 10 to 20 times higher than that of CLB (Bun, et al., 1990). The concentration of CLB is proportional to the dose in an individual

patient but exhibits great inter-individual variation. However, the concentration of NDCB is not proportional to the CLB dose but also exhibits great inter-individual variation (Guberman, et al., 1990; Bardy, et al., 1991). The concentration of CLB and NDCB in the brain is 15% lower than that of their respective steady state concentrations (Arendt, et al., 1987). The lipophilicity of NDCB is lower than that of CLB. The affinity of NDCB for GABA A receptors is 10 fold lower than that of CLB (Greenblatt, et al., 1983). Therefore, the impact of NDCB on the efficacy and adverse drug effects of CLB is unpredictable.

1.2.3. Metabolism

CLB is metabolized to at least 12 metabolites. Its major metabolic pathway is de-alkylation to NDCB, mediated by CYP2B6, CYP3A4 and CYP2C19 as demonstrated using *Baculovirus*-expressed P450s (Giraud, et al., 2004). CLB is also converted to 4'-hydroxyclobazam by CYP2C19. NDCB is primarily metabolized by CYP2C19, to 4'-hydroxydesmethylclobazam. 4'-hydroxyclobazam is de-methylated to 4-hydroxydesmethyl clobazam, mainly by CYP2C9, CYP2B6 and CYP3A4. (Giraud, et al., 2004) (Figure 1.4). This is further metabolised by conjugation and excreted in bile as glucuronate, and in bile and urine as a sulphate.

CYP3A4 and CYP2C19 are the main enzymes involved in the demethylation of CLB, since the amount of CYP2B6 in human liver is much less (0.2%) (Shimada, et al., 1994). CYP2C19 is the main contributor to the hydroxylation of NCLB to OH-NCLB. The hydroxylation pathway of CLB is minor, since patients treated with CLB have a high concentration of NDCB, but hydroxyclobazam is undetectable (Giraud, et al., 2004). Thus, CYP2C19 is the main enzyme involved in the metabolism of CLB and its metabolite NDCB. Therefore, polymorphisms in *CYP2C19* could affect the

metabolism of CLB. This could lead to high or low concentrations of NDCB and CB, affecting the efficacy and adverse effects of CLB (Contin, et al., 2002; Giraud, et al., 2004). Patients homozygous for deficient *CYP2C19* alleles lack enzyme activity (De Morais, et al., 1994). Studies have shown that these patients have a higher concentration of NDCB and are at a higher risk of adverse effects (Contin, et al., 2002; Seo, et al., 2008).

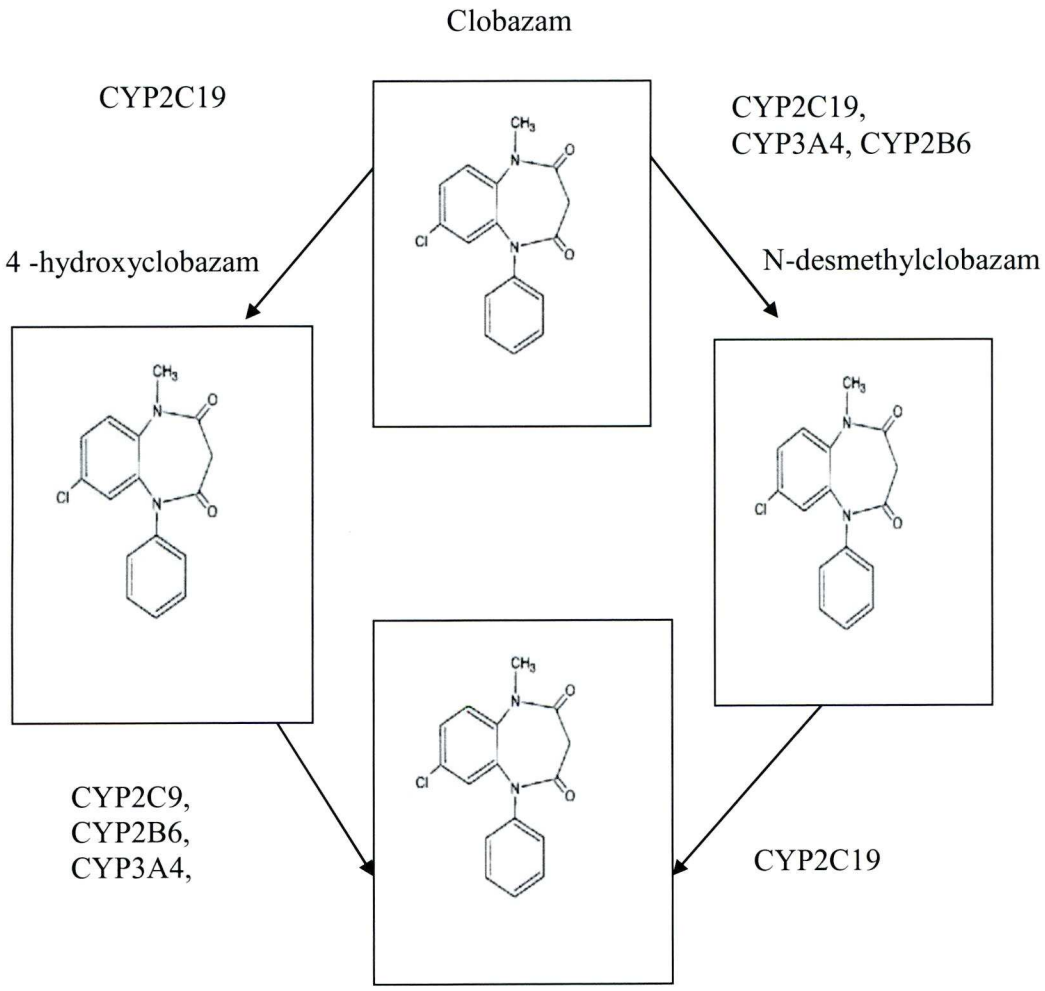


Figure 1.4. Metabolism of clobazam

1.2.4. Adverse drug effects

Tiredness, mild and transient drowsiness, particularly with the initial dose, nausea, dizziness, ataxia and weight gain, disturbed sleep cycles and muscle weakness are common complaints. Side effects are more prevalent in patients with comedications with other AEDs or other medications, than with monotherapy with CLB (Dalby, 2004). Side effects have been reported in clinical trials and only 5 to 15% of these adverse effects are of importance leading to a change in dose or termination of treatment (Koeppen, 1985; Koeppen, et al., 1987). The incidence of tolerance varies among studies from 7.5% (Canadian Study Group for Childhood Epilepsy, 1998) to 77% (Allen, et al., 1983) . Tolerance occurs in patients on high doses of CLB and it can be reduced by intermittent treatment (Feely and Gibson, 1984).

1.2.5. Drug interactions

The pattern of drug interactions with CLB is complex and shows great inter-individual variability. CLB can either increase or decrease the levels of phenobarbital, phenytoin, carbamazepine and valproate (Dalby, 2004). It can lead to worsening of side effects with these drugs, when it increases their levels. The concentration of CLB is generally lower and that of NDCB is higher in patients with inducer comedications. However, there are also reports of raised CLB concentration with phenobarbital, carbamazepine and phenytoin (Wang, et al., 1993). Alcohol and cimetidine significantly increase the levels of CLB (Dalby, 2004).

1.2.6. Uses

Patients of all ages, aetiologies and seizure types have responded to CLB. Patients with non-convulsive status startle seizure, Lennox-Gastaut syndrome and alcoholic

withdrawal symptoms have responded well to CLB (Dalby, 2004). However, very few studies have used CLB as monotherapy. It has been used effectively in CBZ resistant epilepsy and in benign childhood partial epilepsy. It has been reported that CLB is the drug of choice in intermittent usage such as acute epilepsy, in cluster seizures, in serial seizures and as prophylaxis (e.g. on days when it's important to avoid seizures such as traveling, taking examinations, interviews, etc.) (Dalby, 2004). The main drawback of CLB is the potential to develop tolerance. As mentioned above, the incidence of tolerance varies among studies. There is a good correlation between the dose and plasma concentration of CLB, but there is large inter-individual variability (Guberman, et al., 1990; Bardy, et al., 1991). Polymorphisms involved in drug targets, metabolising enzymes, distribution or elimination processes could be responsible for this. Identification of these polymorphisms could enable us to identify patients susceptible to tolerance, and prescribe the right dose for patients, avoiding adverse effects.

1.3. Vigabatrin

Vigabatrin (VGB) is known as a designer molecule since it was synthesized as a structural analog of GABA to specifically inhibit the GABA-T, thus increasing the levels of GABA in the brain (Lewis, 1989). GABA is the major inhibitory neurotransmitter in the brain, and GABA-T metabolises GABA in the brain and tissues of the body (Lippert, et al., 1977) . VGB was first introduced in the UK in 1989. VGB is a racemic mixture of R- and S+ enantiomers. Only the S+ enantiomer is biologically active (Duncan, 1994).

1.3.1. Mechanism of action

VGB forms a covalent bond with GABA-T and specifically inhibits GABA-T in a non-competitive fashion. It increases the levels of brain GABA in rodents and humans (Lippert, et al., 1977). This inhibition peaks 3-4 hours after administration and is maintained for at least 24 hours. Magnetic resonance spectroscopy showed that in clinically used doses, VGB increased the concentration of GABA by 200% in epilepsy patients (Rothman, et al., 1993). Glutamic acid decarboxylase (GAD), the enzyme which synthesizes GABA, is decreased *in vivo* (Jung, et al., 1977). This might be the consequence of negative feedback of the raised brain GABA concentration. VGB also increases β-alanine (a substrate of GABA-T). Since the whole enzyme activity is lost, other substrates of GABA-T can accumulate. (Schechter, et al., 1984; Menachem, et al., 1988). This is further proof that VGB is a non-competitive inhibitor of GABA-T.

1.3.2. Pharmacokinetics

VGB has a favorable phamacokinetic profile. It is almost completely absorbed from the gastrointestinal tract, attaining a peak plasma concentration in 2 hours. Its half life is 5-8 hours in adults and almost 5.5 hours in children. The half life is slightly reduced in the presence of antiepileptic inducer co-medications (Browne, et al., 1987). It has a large volume of distribution (0.8L/kg) in the body. The concentration of VGB in brain is 10% of that in the blood (Menachem, et al., 1988). VGB is mainly excreted by the renal route. Therefore patients with renal impairment and elderly patients experience a slower rate of elimination (Browne, et al., 1987).

1.3.3. Metabolism.

VGB is hardly metabolised in the liver and its protein binding is negligible. It is eliminated relatively unchanged in the urine. Two minor metabolites that constitute less than 5% of the drug are detected in the urine (Durham, et al., 1993).

1.3.4. Drug interactions

No drugs appear to have a pharmacokinetic or pharmacodynamic effect on VGB. VGB can reduce the levels of phenytoin by 20-30%. It does not induce the metabolism of phenytoin. The mechanism of this interaction is not known. There are no other significant drug interactions (Duncan, 1994).

1.3.5. Adverse drug effects

Sedation, dizziness and headache are the most common adverse affects. These symptoms are usually self-limiting. By gradually increasing the dose to 3gms per day over 5 weeks, these symptoms can be avoided. Allergic skin reactions are rare (Duncan, 1994).A literature review of the double blind placebo controlled clinical trials of VGB as add on therapy for refractory partial epilepsy revealed that patients treated with VGB had a higher incidence of depression and psychosis (Levinson and Devinsky, 1999).

It is now established that VGB can cause peripheral bilateral concentric visual field defects (Eke, et al., 1997; Wild, et al., 2007). The nasal field is affected more than the temporal field (Krauss, et al., 1998; Miller, et al., 1999). Although it was initially thought that only peripheral visual field defects result from the use of VGB, central retinal changes have also been reported. They include colour vision disturbances, and reductions in contrast sensitivity and visual acuity (Eke, et al., 1997; Krauss, et al.,

1998; Nousiainen, et al., 2000a; Nousiainen, et al., 2000b). Electrophysiological studies have also demonstrated defects in both the outer and inner retina at the level of the Müller cells (Coupland, et al., 2001). The severity can vary from mild to severe. Males and smokers have a higher risk of developing vigabatrin-induced visual field defects (VVFD). Though there were conflicting reports on the risk factors for VVFD, Wild et al.(2007) in a prospective study, showed that VVFD was associated with duration and mean dose of VGB therapy (Wild, et al., 1999; Wild, et al., 2007). VVFD has an insidious onset. The earliest period at which VVFD has been documented in complex partial seizures is 11 months. VVFD appears on an average after 5.5-8 yrs treatment on VGB (Ovation Pharmaceuticals, 2007).

1.3.6. Uses

VGB has been demonstrated to be effective in a wide range of patients with different types of epilepsies. Significant (more than 50%) seizure reduction was seen in 60-70% patients treated with VGB for complex partial seizures. In addition, 8% of patients with complex partial seizures refractory to anticonvulsants like barbiturates, phenytoin, carbamazepine and valproic acid, attained seizure freedom (Loiseau, et al., 1986; Bruni, et al., 2000). It is the drug of choice for infantile spasms (Chiron, et al., 1990; Chiron, et al., 1997).

However after the report of VVFD in 1997, this drug is only prescribed to patients refractory to other AEDs (Eke, et al., 1997; James, et al., 2009). Regular retinal function assessment and visual field testing is recommended during the treatment period. Polymorphisms in the pharmacodynamic or pharmacokinetic machinery could lead to the development of adverse effects. Identification of these polymorphisms

could enable clinicians to prescribe this drug safely to patients who are not susceptible to adverse effects and at the same time responsive to this drug.

1.4. Pharmacogenetics

Individuals respond to drugs in different ways. In some individuals, a desired result is seen. In others, there may be no effect, while in the remainder, untoward effects develop. Age, sex, disease, environmental factors and co-administration of other drugs, can influence the response characteristics. Genetics can be an important factor determining variability in drug response and development of adverse drug reactions (Pirmohamed and Park, 2001). This has lead to the development of the branch of science called phamacogenetics, a study of how genetic differences influence the variability in patients' responses to drugs, and determines drug efficacy and safety for individual patients (Ameen, et al., 2002). Genetics can affect the metabolism, disposition and elimination of drugs, as well as drug targets and accessory pathways involved in their mechanism of action. Thus, pharmacogenetic variation can be divided into two categories depending on the processes involved:

1. Pharmacokinetics, in which the genetic variants in drug transporters and metabolizing enzymes can result in the variation in the distribution and elimination of drugs.
2. Pharmacodynamics, in which genetic variation in drug targets or target pathways alters the effect of the drug. The drug targets or target pathways can be receptors, ion channels, enzymes, immune molecules, transducer and regulatory proteins.

The number of studies that have investigated genetic risk factors in disease far exceeds those examining the variants in drug response and the development of adverse drug reactions. The completion of the human genome project provided an impetus to pharmacogenetics research. Since then, numerous papers have explored genetic factors affecting drug response, toxicity and efficacy. With the development of genome wide approaches, data availability and technologies, the term pharmacogenomics was introduced in 1997 (Meyer, 2004; Brockmöller and Tzvetkov, 2008). Pharmacogenomics explores the role of the entire genome in both disease susceptibility and drug response, in an attempt to identify specific genes that are associated with diseases and varied drug response. Pharmacogenomics differs from pharmacogenetics in three ways:

(a) Genetic drug response profiles: Pharmacogenetic studies investigated the association of one or a few SNPs involved in one gene or a few candidate genes. This usually focused on pharmacokinetic genes. Thus, identification of variation in drug metabolizing enzymes could lead to selection of appropriate drugs at the correct dose. However, there are 3-12 million SNPs in the human genome which can contribute to variable drug response. Pharmacogenomics envisages identifying the genetic drug response profile in individuals.

(b) Gene expression profile: Pharmacogenomics also allows the analysis of the expression of genes in the human genome in an attempt to investigate the relative contributions of environmental and genetic factors to varied drug responses and disease states.

(c) Drug development: Pharmacogenomics also allows the identification of all genes and all protein products that will help in elucidating the factors causing, modifying or contributing to disease states. This results in the recognition of genes and proteins

involved in the pathogenesis of diseases, which could serve as novel drug targets resulting in alleviation or prevention of disease symptoms (Meyer, 2004). Currently pharmacogenetics and pharmacogenomics are used interchangeably in the public domain, which could lead to confusion.

1.4.1. Genetic variations in human genome

What are the genetic variations affecting drug response and disease status? SNPs are the most common genetic variations. The approximate frequency of SNPs is one in every 300-3000bp, accounting for approximately 3-12 million SNPs. This constitutes <1% of the 3.2 billion base pairs (bp) of the haploid genome (Meyer, 2004; International HapMap Consortium, 2005). Most of the pharmacogenetic studies have used SNPs to explore variable drug responses. Around 1000,000 insertions and deletions are also present in the human genome. Variable number of tandem repeat (VNTR) also add to the genetic variation. For example, an association was found between a 16 amino acid repeat (48 bp) in the dopamine receptor gene and catatonic schizophrenia (Kaiser, et al., 2000). The human genome project revealed that there are around 1500 large genomic segments in variable copy numbers (International HapMap Consortium, 2005).

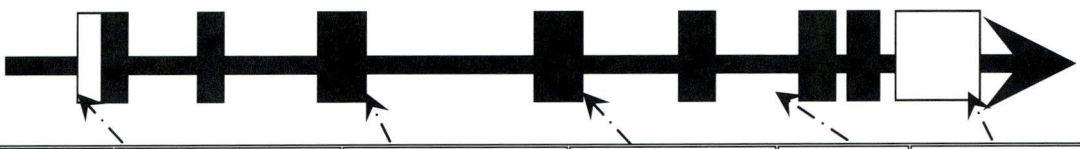
Heritable changes in gene expression can also occur without any change in DNA sequence, as in epigenetics (Eckhardt, et al., 2006). Three interacting processes are important in epigenetics: DNA methylation, modification of histones in chromatin and RNA mediated regulation of gene expression (Peedicayil, 2008). DNA methylation can affect the level of expression of genes and this could contribute to variable drug response.

Table 1.4. Different types of inter-individual variations in human genome.

Genetic variation	Abbreviation	Description	Frequency in human genome
Single nucleotide polymorphism	SNP	Typically two different nucleotides (biallelic SNPs) at one defined position, but more rarely also triallelic variants occur.	12,000,000
Insertion /Deletion	InDel	Deletions (or insertions, depending on the allele frequencies) of between 1 to 1000 nucleotides. More frequent are deletions of one or three basepairs.	> 1,000,000[a]
Varying number of tandem repeats	VNTR	Microsatellites, also termed short tandem repeat (STR) polymorphisms are typically tandem repeats of two, three or four nucleotides, but repeats up to ten nucleotides in length may also be classified in this group. Minisatellites are VNTR polymorphisms in which 10–100 nucleotides are repeated in variable numbers. Repeated segments often do not have exactly identical sequences. VNTRs with larger repeat units (100–1000 bp) are termed satellites.	> 500,000[a]
Copy number variation	CNV	Inheritable deletion or multiplication of DNA segments larger than 1 kb. Currently, about 1500 CNVs distributed through all chromosomes are known; estimated to cover 12% of the human genome length.	> 1500 loci covering 12% of the genome
Epigenetic and somatic variations of the human genome			
Cell karyotype and somatic mutations		Typically in tumours where DNA recombination and repair machineries are damaged, but also in some inherited diseases.	
DNA methylation		Methylation of the cytosine residues of CpG repeats (known as CpG islands) of DNA transmitted through generations. Methylation of CpG islands located in the promoter region of the genes causing down-regulation usually.	> 20% of all genes

a - based on databases and publications (International HapMap Consortium, 2005).
Adopted from (Brockmöller and Tzvetkov, 2008).

What are the possible functional effects of these variations? The above mentioned genetic variations could occur in any part of a gene. Depending on the position in the gene, this genetic variation could alter the function of the genes. Figure 1.5. shows the various parts of a gene and the predictive functional effects of the genetic variations in each of these parts (Brockmöller and Tzvetkov, 2008).



Functional location	**Promotor cis regulatory elements**	**Coding non-synonymous**	**Coding synonymous**	**Intronic**	**UTR**
Functional effects:	Transcriptional regulation of gene expression	Protein activity, Protein stability, Protein interactions	RNA stability, RNA splicing, Translation efficiency	RNA splicing	RNA stability, Translation efficiency (e.g. by miRNA binding)

Figure 1.5. The effect of SNPs in various part of the gene
Adapted from Brockmöller and Tzvetkov, 2008.

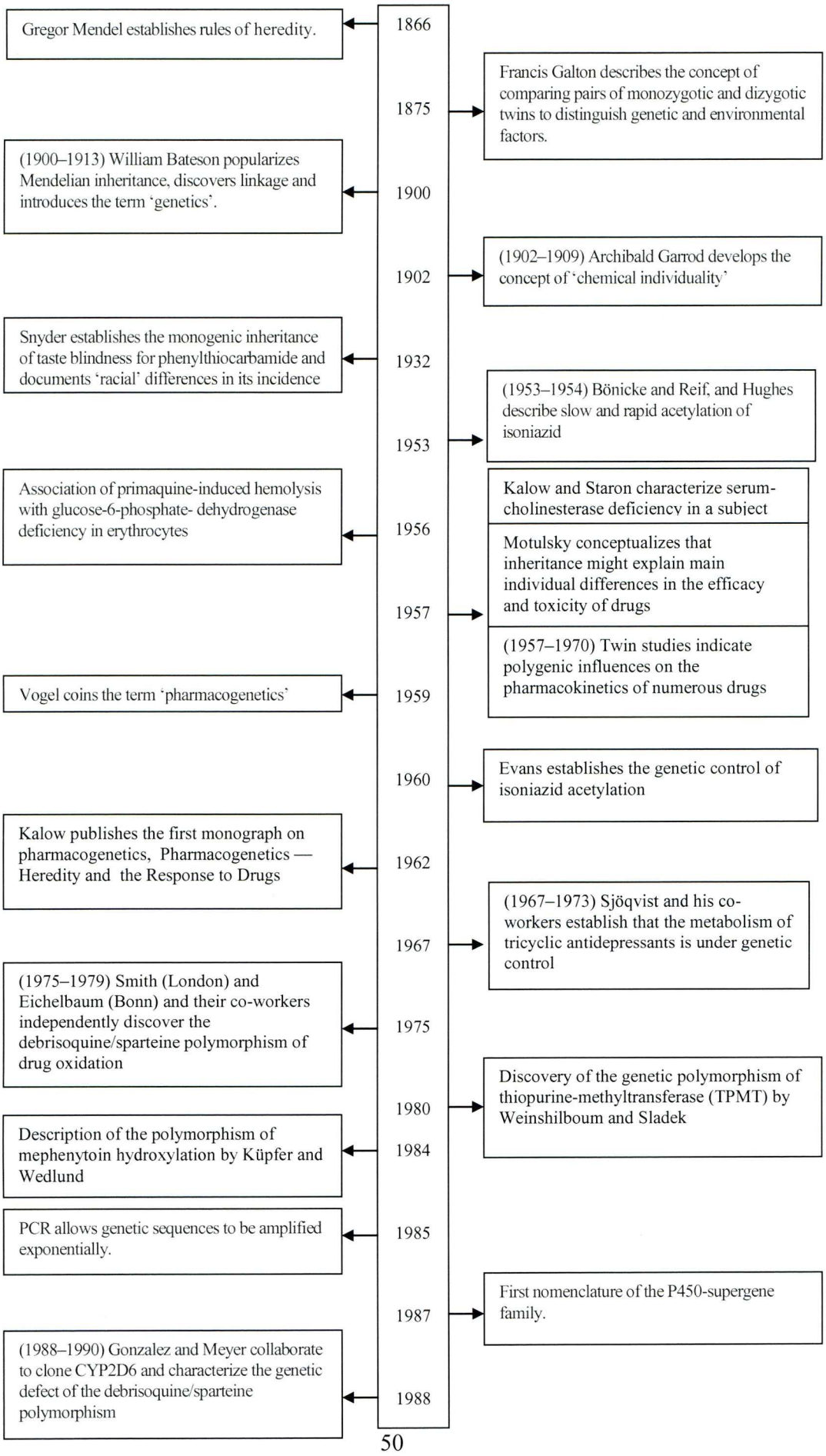
1.4.2. History of pharmacogenetics

Pharmacogenetics has a rich history. Its development is closely related to the advances in the field of human genetics and genomics, molecular pharmacology and modern drug therapy. The major developments of pharmacogenetics are depicted in Figure 1.6.

1850 to 1910: During this time, physiological chemists ascertained that drugs and exogenous chemicals are metabolised by the human body. In the 1860s, Mendel discovered the fundamental laws of heredity. Unfortunately, his findings were not

appreciated until 1900 when they were re-discovered. In 1870s, Ehrlich and Langley proposed that drugs mediate their actions through receptors. These three discoveries laid the foundation for pharmacogenetics (Weber, 2001).

1910 to 1950: During this time, scientists gained insight into the important findings mentioned above. Archibald Garrod and William Bateson from England and Leucien Cuenot in France proposed that genetics played a vital role in the metabolism of exogenous substances. Archibald Garrod, from his observation of patients, with alkaptonuria, and porphyria caused by sulphonal, identified the genetic predisposition to alkaptonuria. He proposed the concept of chemical individuality and thought that enzymes detoxify or metabolize drugs and other exogenous chemicals (Weber, 2001). He elegantly laid down the principles of pharmacogenetics, when he said, "Even against chemical poisons taken by mouth, or by other channels, there are some means of defence. Every active drug is a poison, when taken in large enough doses; and in some subjects, a dose, which is innocuous to the majority of people, has toxic effects, whereas others show exceptional tolerance of the same drug. Some chemical poisons are destroyed in the tissues, provided that the dose given be not too large, and others are combined up with substances to hand, and so rendered innocuous and got rid of" in his book 'The Inborn Factors of Disease' (Garrod, 1931). Despite his insight into genetic basis of drug metabolism, N. H. Snyder conducted the first pharmacogenetics study. A.L.Fox was trying to synthesize a sugar substitute. He noted that people reported that phenylthiocarbide had a bitter taste while others opined that it had 'slight taste' or "taste of sand" (Fox, 1932). In a large study of 800 families, Snyder showed that taste blindness was an autosomal recessive disorder and its frequency varied among populations of different ethnic origins (Snyder, 1932). This study heralded the era of pharmacogenetic studies.


1866
Gregor Mendel establishes rules of heredity.
1875
Francis Galton describes the concept of comparing pairs of monozygotic and dizygotic twins to distinguish genetic and environmental factors.
1900
(1900–1913) William Bateson popularizes Mendelian inheritance, discovers linkage and introduces the term 'genetics'.
1902
(1902–1909) Archibald Garrod develops the concept of 'chemical individuality'
1932
Snyder establishes the monogenic inheritance of taste blindness for phenylthiocarbamide and documents 'racial' differences in its incidence
1953
(1953–1954) Bönicke and Reif, and Hughes describe slow and rapid acetylation of isoniazid
1956
Association of primaquine-induced hemolysis with glucose-6-phosphate- dehydrogenase deficiency in erythrocytes
1957
Kalow and Staron characterize serum-cholinesterase deficiency in a subject
Motulsky conceptualizes that inheritance might explain main individual differences in the efficacy and toxicity of drugs
(1957–1970) Twin studies indicate polygenic influences on the pharmacokinetics of numerous drugs
1959
Vogel coins the term 'pharmacogenetics'
1960
Evans establishes the genetic control of isoniazid acetylation
1962
Kalow publishes the first monograph on pharmacogenetics, Pharmacogenetics — Heredity and the Response to Drugs
1967
(1967–1973) Sjöqvist and his co-workers establish that the metabolism of tricyclic antidepressants is under genetic control
1975
(1975–1979) Smith (London) and Eichelbaum (Bonn) and their co-workers independently discover the debrisoquine/sparteine polymorphism of drug oxidation
1980
Discovery of the genetic polymorphism of thiopurine-methyltransferase (TPMT) by Weinshilboum and Sladek
1984
Description of the polymorphism of mephenytoin hydroxylation by Küpfer and Wedlund
1985
PCR allows genetic sequences to be amplified exponentially.
1987
First nomenclature of the P450-supergene family.
1988
(1988–1990) Gonzalez and Meyer collaborate to clone CYP2D6 and characterize the genetic defect of the debrisoquine/sparteine polymorphism

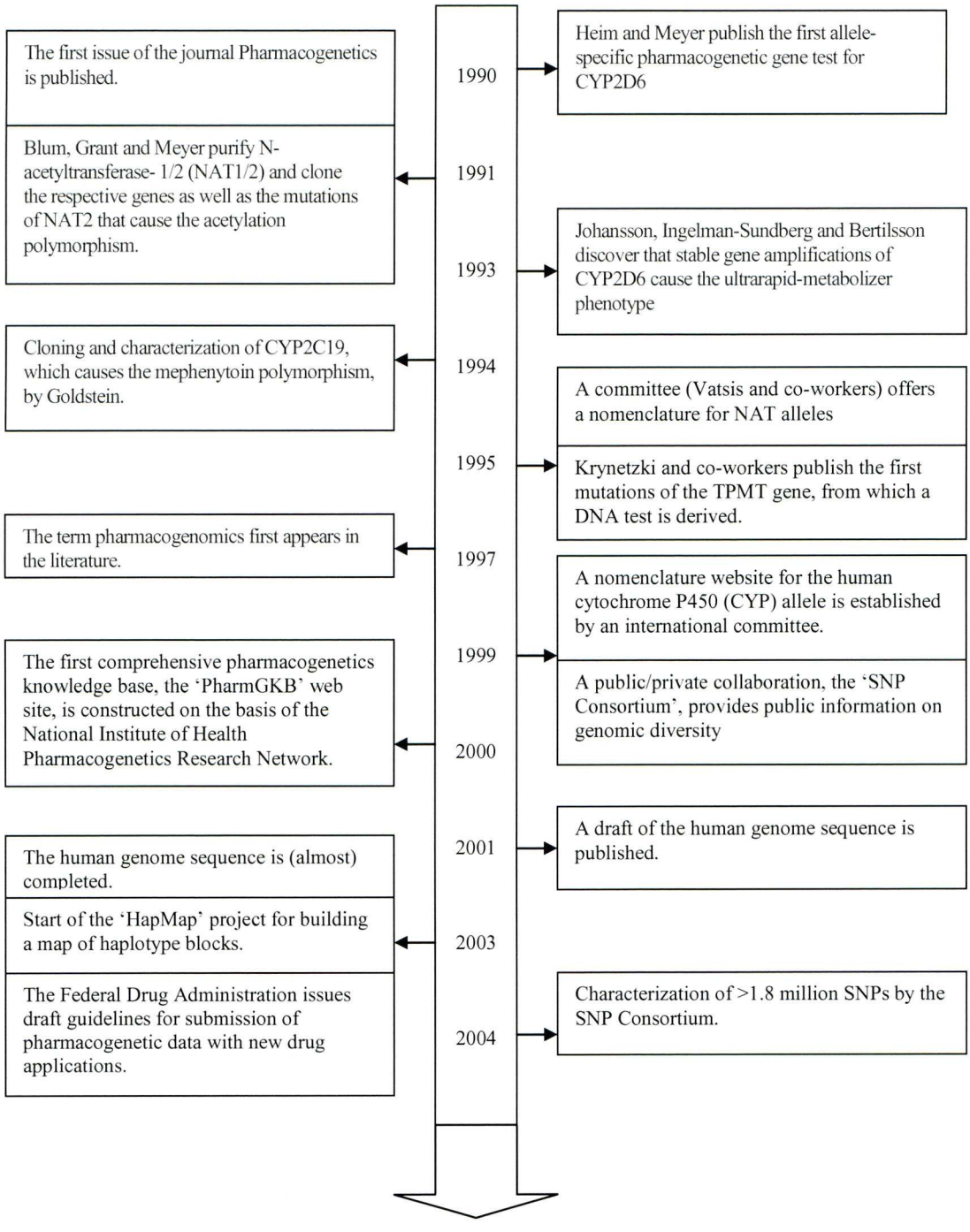


Figure 1.6. Shows the major developments in the history of pharmacogenetics. Adopted from Meyer, **2004.**

1950 to 1990: Pharmacogenetics was established as a discipline during this period. New techniques emerged to accurately measure enzyme activities, drug metabolites and drug responses. Genetics also advanced tremendously during this period. The

double helix structure of DNA was unraveled, human chromosomes were visualized and the biological importance of protein polymorphism was recognized. This provided an excellent opportunity for pharmacologists to study the heritable patterns of metabolism and response to several drugs. By the end of the decade, spanning 1950 to 1960, there were three examples of genetically determined adverse drug reactions to drugs (Meyer, 2004).

During World War 2, it was observed that 10% of African American soldiers developed hemolytic crisis when administered primaquine. This was rare among Caucasian soldiers (Clayman, et al., 1952). Later studies proved that this was due to Glucose-6-phosphate dehydrogenase (G-6PD) deficiency which affected red blood cell (RBC) metabolism (Carson, et al., 1956). The same genetic defect caused hemolysis in some people who consumed fava beans. Pythagoras recognized this in 500 BC (Nebert, 1999). Another paradigm example for pharmacogenetics is prolonged apnoea, up to one hour, from succinylcholine. Altered kinetics in the pseudocholinesterase enzyme, due to genetic variants in the gene which metabolizes succinylcholine were found to be responsible (Lehmann and Ryan, 1956). Family studies showed that pseudocholinesterase deficiency is an autosomal recessive trait (Kalow and Staron, 1957). Though a well studied phenomenon, it took 40 years to prove that peripheral neuropathy in some patients due to isoniazid, an anti tuberculous drug, was due to a polymorphism in its metabolizing enzyme, N-acetyl transferase (Grant, et al., 1992; Vatsis, et al., 1995). Prior family studies had revealed that this trait is autosomal recessive (Evans, et al., 1960).

Arno Motulsky recognized the importance of the above findings. In his seminal paper - 'Drug reactions, enzymes and biochemical genetics', Motulsky described the genetic

basis of adverse reactions to primaquine, succinylcholine and barbiturates. He explained "how hereditary-gene controlled enzymatic factors determine why, with identical exposure certain individuals become sick, where as others are not affected" (Motulsky, 1957). This paper formed the foundation of phamacogenetics. This sparked the interest from many quarters. In 1959, Friedrich Vogel in Heidelburg Germany coined the term pharmacogenetics (Vogel, 1959). In 1962 in his monograph 'Pharmacogenetics: Hereditary and its response to drugs', Kalow reviewed all the articles exploring the genetic effects of drug response (Kalow, 1962). It is also important to note that in 1956, the book "Biochemical Individuality" had a chapter on variability in the response to drugs (Williams, 1956). By 1990, 100 monogenic polymorphic traits in pharmacogenetics had been reported. The majority of these polymorphisms were reported in drug metabolizing enzymes. A community of researchers interested in pharmacogenetics was developing. The first international conference on pharmacogenetics was held at the New York Academy of Sciences in 1967 (Meyer, 2004).

1990-2000: The technical advances in molecular genetics in the 1990s lead to cloning, sequencing and site directed mutagenesis to study the organization and composition of genes. Currently although the sequence variability in genes is becoming known, how this relates to variability in drug response is being explored. The paradigm for the study of pharmacogenetics has also changed. In the last century, pharmacogenetic studies started with the phenotype and were followed by studies of genetic variation. Thus, the earlier pharmacogenetics studies investigated the association between genetic variations in the genes involved in pharmacokinetic processes - so called 'low hanging fruit,' rather than those coding for drug targets (Weber, 2001).

2000-Present: In this century, many studies start with the genotype and are followed by an analysis of the phenotype. Pharmacogenetic studies are also increasingly focusing on the genes for drug targets. For example, Lynch et al. (2004) and Paez et.al. (2004) demonstrated that in patients with somatic gain of function, the response to geftinib was better, secondary to mutations in the epidermal growth factor receptor in non-small lung cell carcinoma. This was the first study to demonstrate the influence of somatic cell mutations to drug response, and this is now increasingly being investigated.

The focus of pharmacogenetic studies is shifting to polygenic variation in both pharmacodynamic and pharmacokinetic processes. In many cases, pharmacokinetic genetic variation alone is unable to explain the variance in phenotypes. A classical example is warfarin. Warfarin is the most commonly prescribed anticoagulant in Europe and North America. In spite of the dosing being monitored by INR, there is a wide variation in warfarin doses and many patients develop bleeding, an important and common adverse drug effect. Warfarin acts on vitamin K epoxide reductase complex subunit 1 (VKORC1) preventing coagulation of blood. It is metabolized by CYP2C9. Many studies have shown that the SNPs in VKORC1 account for 15 to 30% of the variation in dose requirement, while those in CYP2C9 are responsible for 6 to 18 % of the variability in warfarin dosing (Rieder, et al., 2005; Wadelius, et al., 2005; Sconce, et al., 2008). This shows that in the case of warfarin, pharmacokinetic and pharmacodynamic pathways are important in determining drug dose.

1.4.3. Where are we now?

Many genetic association studies have appeared over the last decade. A major issue however is that very few have been replicated. In fact, in 2002, only 6 out of 166

putative genotype-phenotype association studies could be replicated after at least 3 attempts (Hirschhorn, et al., 2001). There are many possible reasons reported for the problems encountered in replication (Hirschhorn, et al., 2001; Ioannidis, et al., 2001; Ioannidis, 2003):

(a) Publication bias: The risk estimates in small studies are subject to large variation and usually only large studies with positive results are published.

(b) Weak genetic effects and lack of power: A large sample size is required to demonstrate a minor effect by genetic variants of complex diseases. Many pharmacogenetic studies have small numbers of patients and are likely to be under-powered.

(c) Population stratification: The minor SNP allele frequencies vary among populations. So if the study population includes subjects from different ethnic groups, like Caucasians, Africans, and Asians, the sample size may not be adequate to detect the effect of SNPs that have variable frequencies in the populations.

(d) Heterogeneity in classification of phenotypes across studies. Poor phenotyping is common in many studies.

(e) Different LD patterns between populations or different SNP markers between studies.

(f) Gene-gene or gene-environmental interaction modifier genes can also influence drug response.

(g) Regional population ethnic differences.

(h) Another functional variant linked to the allele under study.

(i) Unequivocal genotype is defined as the assignment of a genotype by scientific investigators without any room for error. It is becoming increasingly difficult to assign an unequivocal genotype. Allelic heterogeneity, locus (non-allelic)

heterogeneity, large number of genes contributing to a trait, genocopy and synonymous mutations that unpredictably alter the trait can make it difficult to assign an unequivocal genotype (Nebert and Vesell, 2004). New discoveries about the human genome further make it difficult to assign a genotype. Walsh et al. (2006) showed that 12% of the patients tested negative for breast cancer 1 early onset gene (BRCA 1) and breast cancer 2 early onset gene (BRCA 2) SNPs, in a vast array of DNA tests. However, these patients had gene rearrangements not included in the DNA tests.

Risch and Merikangas (1996) raised concerns about the ability of linkage studies to detect genes of modest effect or lower penetrance, which is the case in complex diseases. They proposed that genome wide association studies that tested all candidate genes, and perhaps all genes in the genome, would have greater power to detect genes of modest effect. The first ever genome wide association study (GWAS) was published in 2002, where an association was drawn between Lymphotoxin-α gene and myocardial infarction. 92,788 gene SNP markers were tested in this study (Ozaki, et al., 2002). By the end of 2007, two dozen GWAS had been reported and many of these had been replicated (Nebert, et al., 2008).

Overall, the quality of pharmacogenetic studies has been steadily improving. There have been many studies that have now proven the association between genetic variants in drug metabolizing enzymes and drug transporter genes and pharmacokinetics of at least one drug, and have also been replicated. 10% of the new drugs introduced into the market today carry pharmacogenetic information in their labels. More and more pharmacogenetic tests are being approved by the Food and Drug Administration (FDA) and are available commercially. However, translating

pharmacogenetic principles to clinical practice remains a challenge (Swen, et al., 2007).

1.4.4. Translation of pharmacogenetics into the clinic

There are various challenges in translating pharmacogenetics into clinical practice as shown in Figure 1.7 (Swen, et al., 2007):

(1) Lack of scientific evidence for improvement in patient care by pharmacogenetic testing.

There is a paucity of large powered genotype-phenotype association studies. Most of the currently available studies are under powered to detect real associations. In addition, many of these are single dose studies done in healthy volunteers. These are inadequate since the pharmacokinetic or pharmacodynamic processes are altered in the patients on chronic treatment. Therefore, the findings from these studies cannot be used as a basis for adopting pharmacogenetic tests in clinical practice. Moreover, a large proportion of the studies have not explored the association of genotypes and clinical endpoints like efficacy or adverse drug reactions. They have explored the effects of genotypes on the pharmacokinetic parameters of the drugs. The wide variation in pharmacokinetic parameters may not translate into clinical effects.

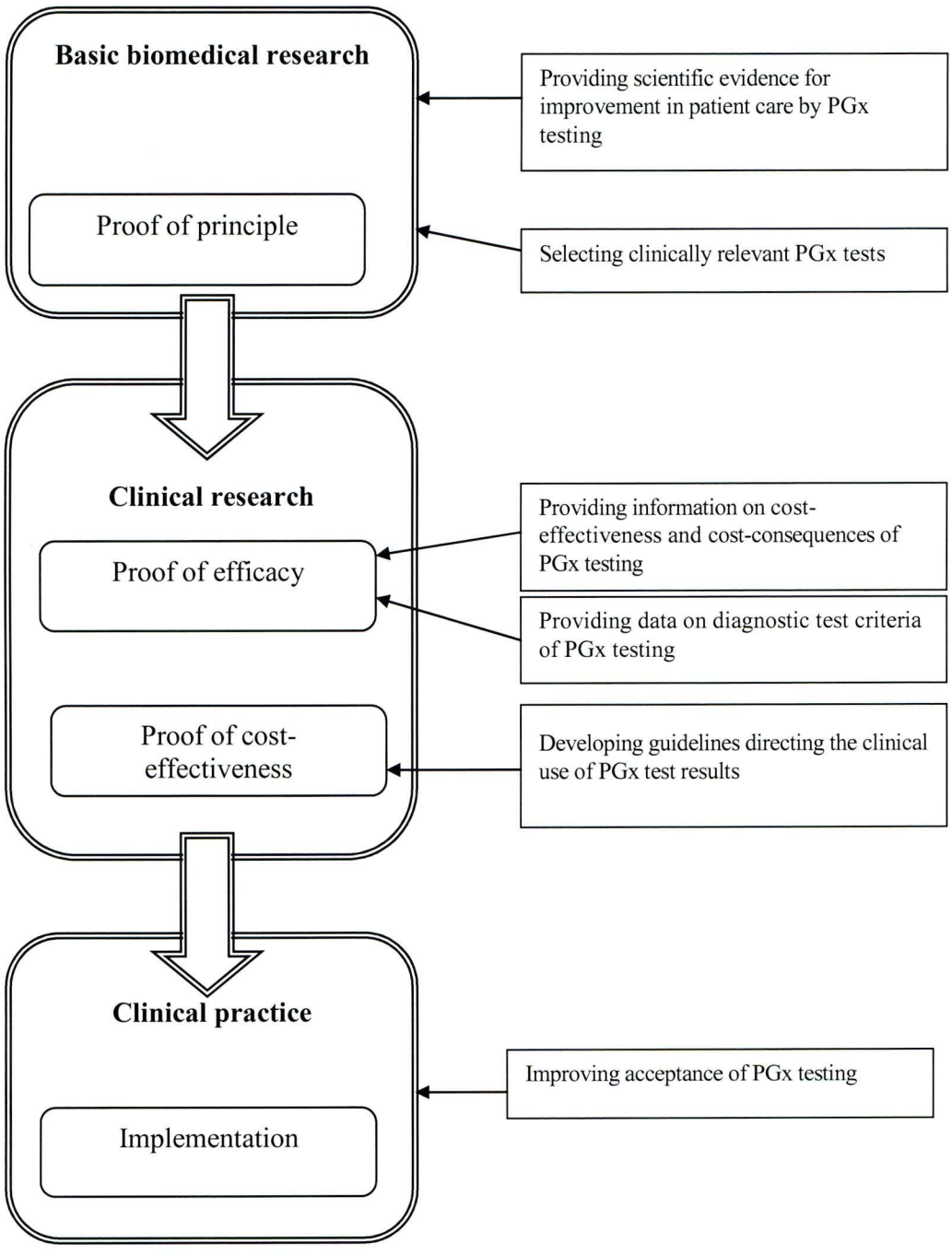


Figure 1.7. Different phases & associated challenges on the road to clinical implementation of pharmacogenomics. PGx – Pharmacogenetics. Adopted from (Swen et al. 2007)

For instance, a wide variation exists in the plasma levels of nortryptyline among patients with mutant alleles of CYP2D6. However, even an increase of 10 fold in

drug levels from the population norm does not necessarily result in toxicity (Swen, et al., 2007).

(2) Selection of clinically relevant pharmacogenetic tests

Some genotype-phenotype associations might be true and replicable. However, their clinical relevance is questionable. For example, the variation in CYP2D6 that results in the ultra-rapid metabolizer (UM genotype) status is relatively rare in the Caucasian population. The UM genotype results in reduced levels of 5-hydroxytryptamine receptor (5HT3 receptor) antagonists such as tropisetron and ondansetron reducing efficacy causing more nausea and vomiting (Kaiser, et al., 2002; Kim, et al., 2003). However since the prevalence of the UM genotype is lower amongst Caucasians, the number of patients needed to genotype in order to prevent one patient from unwarranted nausea and vomiting is 50 (McLeod, 2002). This is economically not viable and hinders the implementation of this pharmacogenetic test (Maitland, et al., 2006).

(3) Diagnostic test criteria for pharmacogenetic testing

A diagnostic test should be able to predict the outcome of drug treatment. It should be sensitive and specific. Drug response is a complex process. Pharmacokinetics, pharmacodynamics and environmental factors can contribute to this. A diagnostic test for single polymorphisms may only account for part of the variability of drug response. The diagnostic test criteria for pharmacogenetics tests should be mentioned to evaluate the clinical usefulness of this test.

(4) Information on cost effectiveness and cost consequences of pharmacogenetic testing

The health insurers will require more information on cost effectiveness of pharmacogenetics diagnostic tests to reimburse routine tests. There are very few cost effectiveness evaluations of pharmacogenetic tests. However, there is little information about which pharmacogenetic tests predict adverse drug reactions or effects, and the cost of genotyping is decreasing. Even though cost effectiveness analysis has not been done for all pharmacogenetic tests before being used in the clinic, any information may aid clinical use of these tests in the future.

(5) Guidelines directing the clinical use of pharmacogenetic test results.

Very few pharmacogenetic studies exist, which recommend dose adjustments according to genotype. Warfarin dose adjustments that are recommended based on polymorphisms in VKORC1 and CYP2C9, and height of patients are an example, but are still limited by the fact that the effectiveness of dosing algorithms has not been tested in clinical settings.

(6) Improved acceptance for pharmacogenetic testing.

It took seven years before the use of the Calvert formulae to optimize the dose of carboplatin became routine. In contrast, testing for human epidermal growth factor receptor 2 (HER2/NEU) over expression was made mandatory by the regulatory authorities to prescribe trastuzumab (for the treatment of breast cancer) within a year. This testing was advocated by the company that manufactured the drug and by patient advocacy organizations. It is possible that if regulatory agencies recommended testing prior to prescribing the drug and this in turn is supported either by pharmaceutical companies or by patient organizations, or both, this will expedite the

process of clinical updates of pharmacogenetic testing. However, the critical issue is the weight of evidence to support genotyping before prescription.

Various stakeholders need to work together to aid this translation into clinical practice. This should include the biotechnology and the analytical industry, the pharmaceutical industry, the research institutions, funding agencies, regulatory agencies, clinicians and patients. Each of these stakeholders has important individual and collective roles in developing and executing the clinical application of pharmacogenetics. Fast, reliable and affordable assays for routine pharmacogenetic testing should be developed by the biotechnology and analytical industry. The response of industry to pharmacogenetics tests has been reserved (Swen, et al., 2007). A 2001 report stated that investments to develop a drug would be reduced by 300 million US dollars and by 2 years, if pharmacogenetic testing is used in the development of drugs (Tollman, et al., 2001). The FDA is encouraging voluntary pharmacogenetic data submission. European agencies for evaluation of medicinal products and FDA have issued a joint procedure for the voluntary submission of pharmacogenetic data (http://www.emea.europa.eu/pdfs/general/direct/pr/FDAEMEA.pdf). Funding agencies should be more willing to fund clinical trials involving pharmacogenetics. The reimbursement authorities or companies should be willing to pay for pharmacogenetic tests, taking into consideration the medical events they can prevent. Physicians and clinical pharmacists have to be educated on the usage of pharmacogenetic tests. Patients and patient advocacy groups have an important role in implementing pharmacogenetics (Swen, et al., 2007). We are far away from implementing pharmacogenetic tests for CLB and VGB. Polymorphisms specific to patient response, dose and adverse drug reactions are being investigated (Seo, et al.,

2008). Once they are established, the aforementioned hurdles have to be surpassed to bring pharmacogenetic tests specific for CLB and VGB into clinical practice. However, these drugs are not widely used now.

1.5. Aims of the thesis

In view of the points discussed above, for VGB, the hypothesis tested in this thesis was that variation in the ABAT gene, which encodes GABA-T enzyme may modulate GABA-T enzyme activity, and thereby predispose to the visual field defects. For CLB, the hypothesis being tested was that variability in the metabolism of CLB may lead to variation in pharmacokinetics, and drug response parameters.

In order to test these hypotheses, the objectives of this thesis were to

1) Develop a GABAT activity assay using platelets as a non-invasive source of human material.
2) To identify putative functional SNPs in the ABAT gene.
3) To assess the genotype-phenotype correlation of SNPs in the ABAT gene and the activity of GABA-T in platelets.
4) To assess GABA-T activity in patients who developed visual field defects and compare them with patients without visual field defects despite prior exposure to VGB.
5) To develop a HPLC assay to measure the plasma concentrations of CLB and NDCB.
6) To correlate the known SNPs in the *CYP2C19* gene with the dose of CLB, plasma concentrations of CLB, NDCB and their ratios.
7) To identify the predictors of efficacy, tolerance and propensity to develop adverse drug reactions to CLB.

Chapter 2

GABA transaminase – characterisation of phenotype-genotype relationship

2.1. Introduction

GABA-T is involved in the catabolism of the main inhibitory neurotransmitter GABA. It is ubiquitous in distribution, with the highest expression in liver, brain, pancreas and kidney, moderate expression in prostate, testis and thyroid and low expression in heart, ovary, adrenal gland, mammary gland and placenta (De Biase, et al., 1995; Jeon, et al., 2000). It is also present in platelets (White, 1979) and hair follicles (Armijo, 1989). GABA-T is a α2 homodimer with 472 amino acids in each subunit. These subunits are intertwined with pyridoxal phosphate cofactor close to the subunit interface. The subunits are made of 2 identical monomers (De Biase, et al., 1995; Storici, et al., 1999) (Figure 2.1).

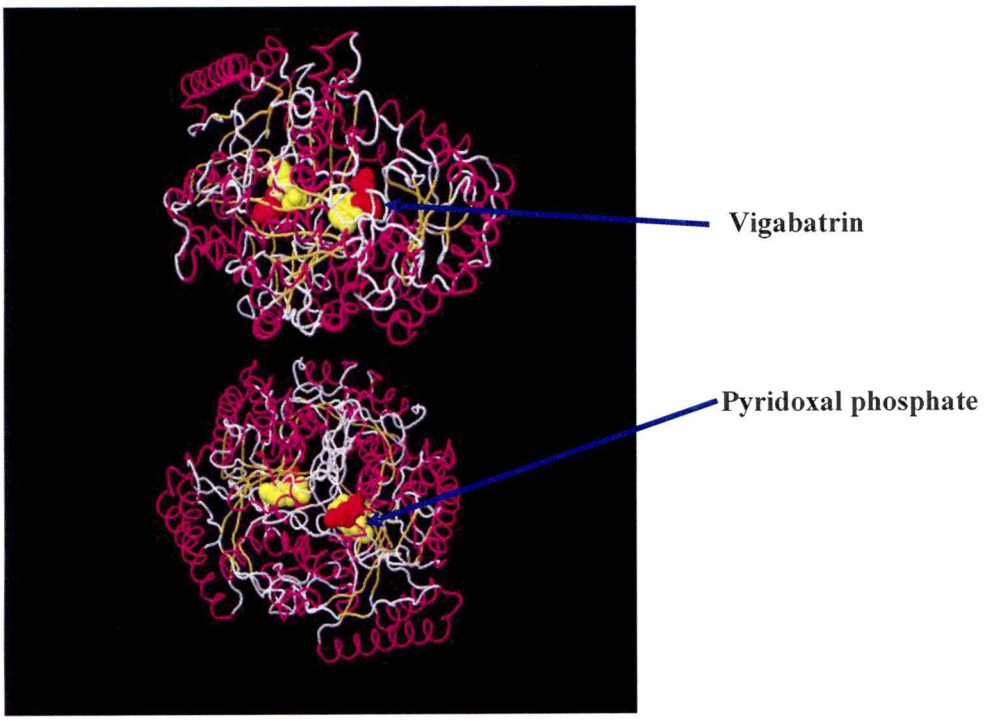


Figure 2.1. Shows the 2 identical monomers of GABA-T with vigabatrin bound to the active sites and its co-factor pyridoxal phosphate

The activity of GABA-T is known to vary. Berrettini *et al* (1982) reported that genetic factors could play a major role in determining platelet GABA-T activity. The 4-aminobutyrate aminotransferase (ABAT) gene encodes GABA-T enzyme. It is located on the short arm of chromosome 16 at 13.2 pter. It has 3 transcripts which code for the same protein (Tsuji, et al., 2010). ABAT deficiency is associated with psychomotor retardation, hypotonia, hyperreflexia, lethargy, refractory seizures, and electroencephalograph (EEG) abnormalities.

ABAT is also a polymorphic gene, with variants that could affect GABA-T activity. To the best of my knowledge, there have not been any studies looking into the effects of SNPs on GABA-T activity.

Platelet GABA-T has previously been used as a model for brain GABA-T. This is because they have comparable kinetic properties, molecular properties and pharmacology (White and Sato, 1978; White, 1979; White and Faison, 1980; Rimmer, et al., 1988) Therefore I have used platelet GABA-T to investigate the functional effects of SNPs on brain GABA-T activity.

There are many methods to estimate GABA-T activity (Pitts, et al., 1965; Gonnard, et al., 1973; Jung, et al., 1977; White, 1979). The spectrophotometric method described by Schousboe et al. (1973) is simple, cost effective and does not involve radioactivity. Therefore, this method was standardised in platelets.

The aims of this chapter were to

1. To standardise a simple, reproducible and cost-effective assay for platelet GABA-T.
2. To select putative functional SNPs in the ABAT gene.

3. To genotype these SNPs in healthy volunteers.
4. To correlate genotype to the activity of platelet GABA-T in healthy volunteers.

2.2. Methods

2.2.1. Materials

Optiprep was purchased from Axon Lab AG, Le Mont-sur-Lausanne, Switzerland. All the other chemicals used for the experiments mentioned in this chapter were purchased from Sigma Aldrich, Poole, England. ABI PRISM 7000® was used to perform real time polymerase chain reaction (PCR). The absorbance of acetylpyridine (dihydronicotinamide adenine dinucleotide –coenzyme Q reductase) NADH was measured by Bio-Tek FL600 fluorescence micro plate reader.

2.2.2. Study population

DNA was extracted from the blood of 32 healthy volunteers of Asian and Caucasian origin. Ethical approval was obtained from the Capenhurst Independent Research Ethics Committee, Bromborough Wirral. Blood was drawn from these healthy volunteers for the GABA-T assay and genotype estimation, after obtaining written informed consent. The study population was limited to this number since there were only these many individuals in the department who consented to donate blood.

For the estimation of the allelic frequency of selected SNPs, DNA of 107 individuals from the DNA bank in our lab (healthy Caucasian controls which have been anonymised) was also used with the above mentioned healthy volunteers. Ethical approval was obtained from the ethical committee mentioned above.

2.2.3. Platelet isolation

GABA-T activity has previously been shown to be reduced after food (Berrettini, et al., 1982; Sherif and Ahmed, 1995). Therefore, I collected fasting venous blood samples in tubes containing ethylenediaminetetraacetic acid (EDTA-100mM) as an anticoagulant (150 µl for 10ml). To optimise the platelet isolation method, GABA-T activity in platelets was estimated under the following conditions. Platelets were isolated (a) soon after blood collection, (b) from plasma, after allowing the blood to stand for 2hrs, and (c) 2 hrs after adding dextran. Dextran was added to help sediment the blood cells.

Platelet isolation was undertaken using the density gradient method described by Bagamery et al. (2005). OptiPrepTM was reconstituted according to the instructions of the manufacturer to obtain a 1.063 gm/ml solution. In a 15 ml centrifuge tube, 5 ml of plasma was layered over 5 ml of the 1.063 gm/ml solution and centrifuged at 350 x *g* for 15 min as shown in Figure 2.2. In order to avoid disturbance to the banded material, the rotor was allowed to decelerate without the brake. The platelet-containing band, extending into the density barrier from just above the interface was harvested (subsequent to the removal of the clear plasma layer). This platelet rich layer was centrifuged at 10,000 x *g* at 4°C. The supernatant was discarded and the pellet from 30ml of blood was re-suspended in 500µl of buffer (pyridoxal phosphate (0.2mM), 2-aminoethylisothiouronium bromide hydrobromide (AET) (1mM), pH 7.2). This was sonicated for 3 cycles of 10s each on ice at 20% efficiency. The platelets obtained were aliquoted and stored at -70°C till the GABA-T activity was measured.

2.2.4. GABA-T assay standardisation

2.2.4.1. GABA-T activity

The activity of GABA-T in the platelet preparations was measured using a spectrophotometric method with GABA as a substrate described by Schousboe et al. (1973). The GABA-T assay consisted of a coupled assay, as shown in Figure 2.3. The first step is the rate limiting reaction. GABA-T converts GABA to succinic semialdehyde, during which α-ketoglutaric acid is converted to glutamic acid. The glutamate formed in the first reaction is converted to α-ketoglutaric acid by glutamate dehydrogenase in the presence of acetylpyridine NAD+. Acetylpyridine NADH is also formed and is measured at 340nm.

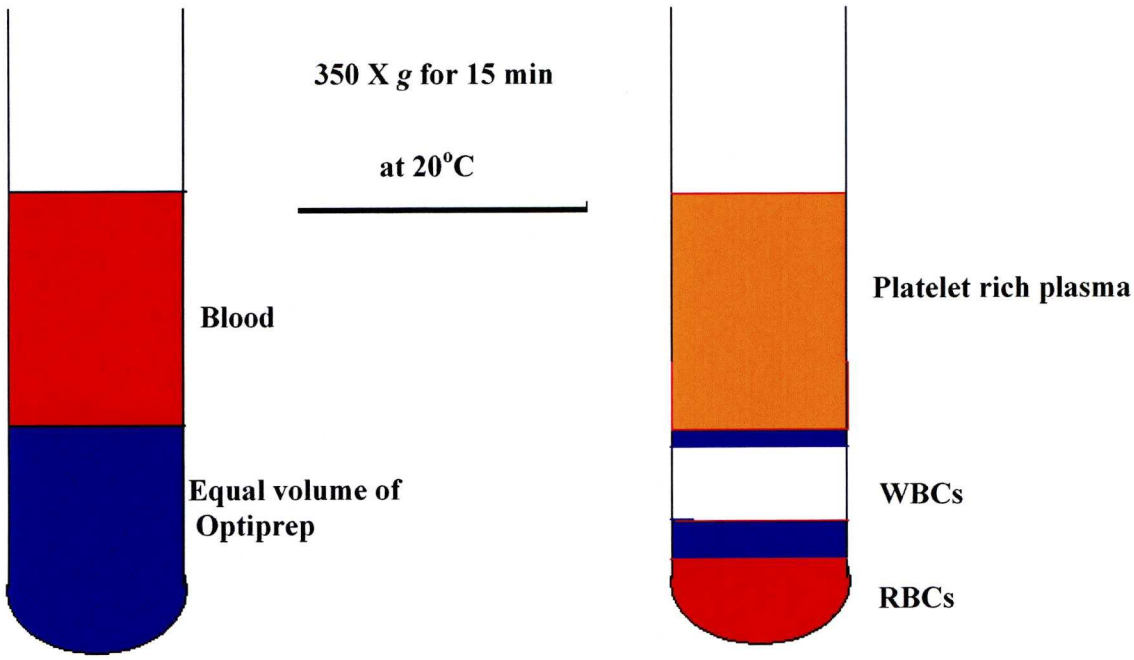


Figure 2.2. Schematic diagram showing platelet isolation using OptiPrep™ as the density gradient

The volume of the first reaction was optimised to 100µl. 20µl of the platelet preparation was added to 20µl of potassium phosphate buffer (pH=8, 50mM). A

mixture of 20µl of 10mM α- ketoglutaric acid, 1µl of 100µM AET, 20 µl of 50mM GABA and 0.20 µl of 20µM pyridoxal phosphate were prepared. This was added to the platelets and the volume made up to 100 µl with water. The mixture was vortexed for 10s and then incubated at 37°C in a shaker for 30 min. Blank samples were incubated with sodium phosphate buffer instead of the platelets. The reaction was terminated by the addition of 2µl of 100µM aminooxyacetic acid and placing the reaction tubes on ice. The second reaction mixture contained 60µl of 50mM potassium phosphate buffer (pH=8), 30µl of 25mM hydrazine, 6µl of 1mM aminooxyacetic acid, 30µl of 750mM acetyl-pyridine-NAD+, and 2µl of 1.3 units/ml glutamate dehydrogenase. The volume was made up to 300µl with water. This was added to the first reaction. After a short vortex, the reaction was run for 90 min at room temperature and the optical density measured at 340nm. A schematic representation of these two reactions is shown in Figure 2.3. Here I am measuring acetylpyridine NADH produced as a result of reduction of acetyl pyridine NAD+.

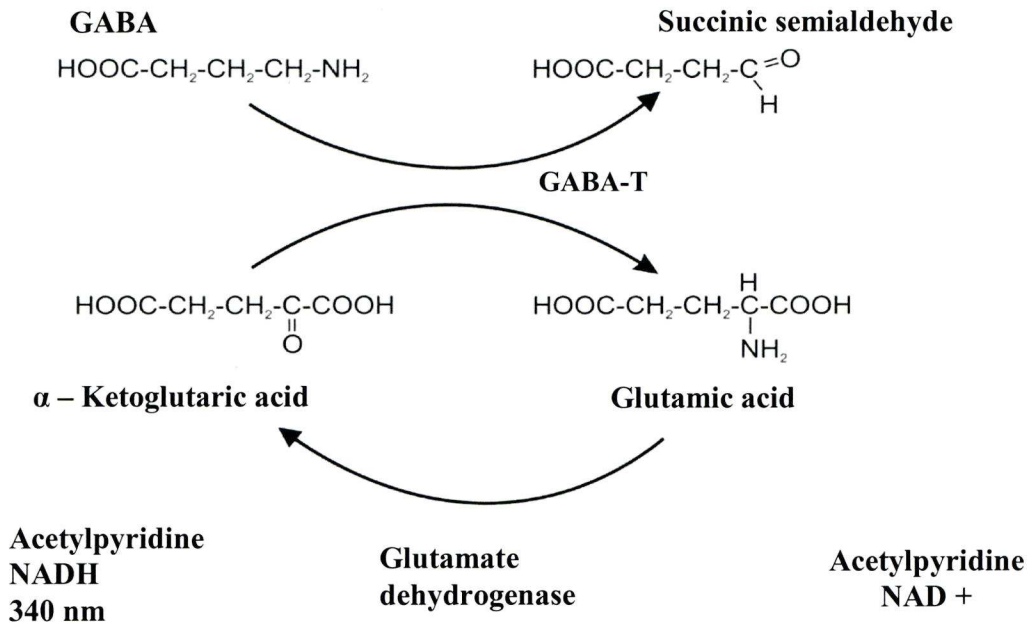


Figure 2.3. Shows the schematic representation of GABA-T assay

The protein concentration in the platelet samples was measured by the Bradford reaction (Bradford, 1976). GABA-T activity was expressed as nm/mg/ml (nanometre/milligram/ml) of protein. Various concentrations (0.2, 0.4, 0.6, 0.8 and 1mg/ml) of bovine serum albumin (BSA) were prepared in a platelet buffer (pyridoxal phosphate (0.2mM), AET (2-aminoethylisothiouronium bromide hydrobromide) (1mM, pH 7.2)) mentioned above. The samples for protein estimation were diluted (1/10, 1/20, 1/40) in platelet buffer. 20 µl of the above mentioned solutions were aliquoted on to a 96 well plate. 250 µl of Bradford reagent was added to each well. The plate was read on a spectrophotometer at 570 nm. The concentration of the protein in samples were calculated from the protein concentration in the BSA.

2.2.4.2. Assessment of storage on GABA-T activity

Platelets were isolated using the above method mentioned in section 2.2.3, and stored at -70°C. Once platelet preparations were taken out of -70°C freezer, they were always kept on ice till used for the assay. After one week of storage, GABA-T activity was measured and was compared to that of platelets stored for one day.

2.2.4.3. Measurement of GABA-T activity using a 96 well plate format

The initial reaction volume (during standardisation) for the first reaction was 1,100µl while that of second reaction was 1000µl. The optical density was measured at 340nm by a spectrophotometer. Since it is more cost effective to set up reactions in small volumes, I standardised the reaction volume to 100µl and 300µl, for the first and second reactions, respectively. The assay was read on a 96-well plate by a Bio-Tek FL600 fluorescence micro plate reader at 340nm of absorbance and compared to that done in a larger volume.

2.2.4.4. Assessment of the optimum time required for the 1st reaction

The first reaction in the GABA-T assay is the rate limiting reaction. To estimate the optimum time required for the first reaction, the reaction was stopped at 10, 20, 30, 40, 50, 60, 70, 80 and 90 min. After the second reaction, the OD was checked and compared for all these assays at the different time points.

2.2.4.5. Effect of thawing on platelet GABA-T activity

GABA-T activity was determined at 30, 60, 90, and 150 min after taking the platelets out of the -70°C freezer. Platelet preparations were always kept on ice till they were used for the GABA-T assay. This gave me an indication of the optimum time at which the platelets are active after removal from the -70°C freezer.

2.2.4.6. Inhibition of GABA-T activity by vigabatrin (VGB)

Platelets were incubated with VGB for 10 min (Jacob, et al., 1990) . GABA-T activity was then assessed as outlined above. Various concentrations (3μM- 3mM) of VGB were used to attain a dose response curve.

2.2.5. Prediction of putative functional SNPs in the ABAT gene

SNPs were selected from public databases like dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez), Ensemble (http://www.ensembl.org/index.html) and UCSC (http://genome.ucsc.edu/) with the help of SNP prediction software programs, PupaSNP (Conde, et al., 2004) and SNPper (Riva and Kohane, 2004). The inclusion criteria used were (a) location of the SNP (exons, promoter region, exon-intron boundary, 3'untranslated region, 5' UTR), (b) minor allele frequency > 5%, (c) population (Caucasian), (d) presence of SNP in conserved regions of the gene. Sequences of at least 100 bp and a percent identity of at least 70% that match in alignments of sequences between two species were considered as conserved region. SNPs in this region could alter the function of the gene (Loots, et al., 2000) and (e) function. SNPs are known to alter the function of the gene products-proteins. They could increase (Sim, et al., 2006) or decrease the activity of enzymes (De Morais, et al., 1994).

2.2.6. Genotyping

All samples were genotyped using the TaqMan allelic discrimination assays according to the manufacturer's instructions on the ABI PRISM 7000® platform. TaqMan chemistry (Figure 2.4) was used for allelic discrimination. Briefly, an oligonucleotide was constructed consisting of a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. When the probe is intact, the energy from the

fluorescent dye flows to the quencher dye by a phenomenon named fluorescence resonance energy transfer (FRET). In the presence of a target sequence, the 5' nuclease activity of the Taq DNA polymerase cleaves the annealed probe, separating the reporter dye from the quencher resulting in fluorescence of the reporter dye. This fluorescence can be measured and quantified. More reporter dye is cleaved from its probe resulting in an increase in fluorescence with each cycle of the PCR. This fluorescence is proportional to the amount of PCR product.

In allelic discrimination PCR assays, a specific fluorescent dye labeled probe was used for each allele. The probes contained the 6-FAM and VIC dyes, to differentiate the amplification of the two alleles. During PCR, each probe annealed to the complimentary sequence between the forward and reverse primers. Taq DNA polymerase only cleaved the hybridized probes. The reporter dye was cleaved from the quencher dye. The fluorescent signal generated by the PCR reaction indicated the allele present in the sample, as demonstrated in Figure 2.5.

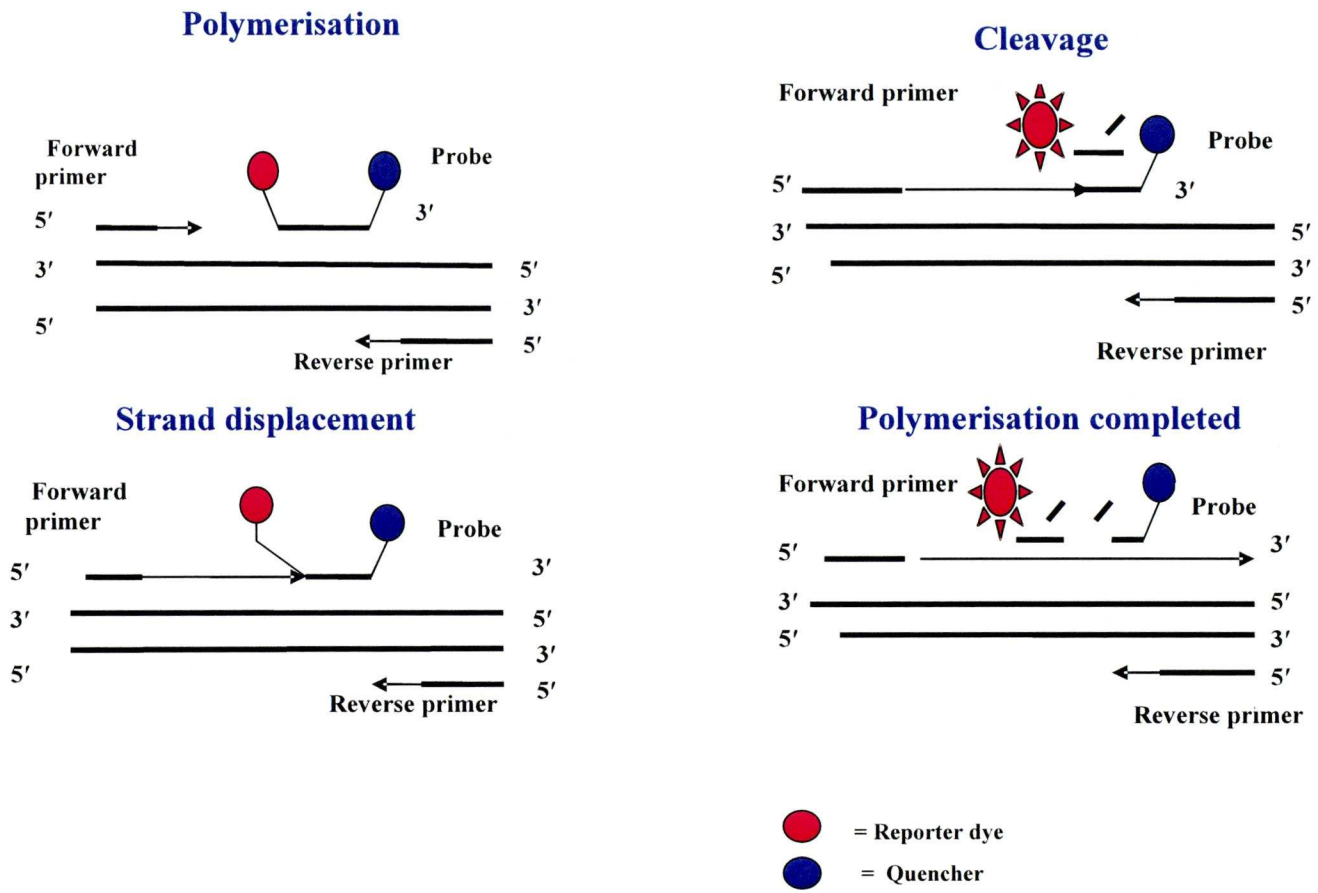


Figure 2.4. A schematic representation of Taqman chemistry

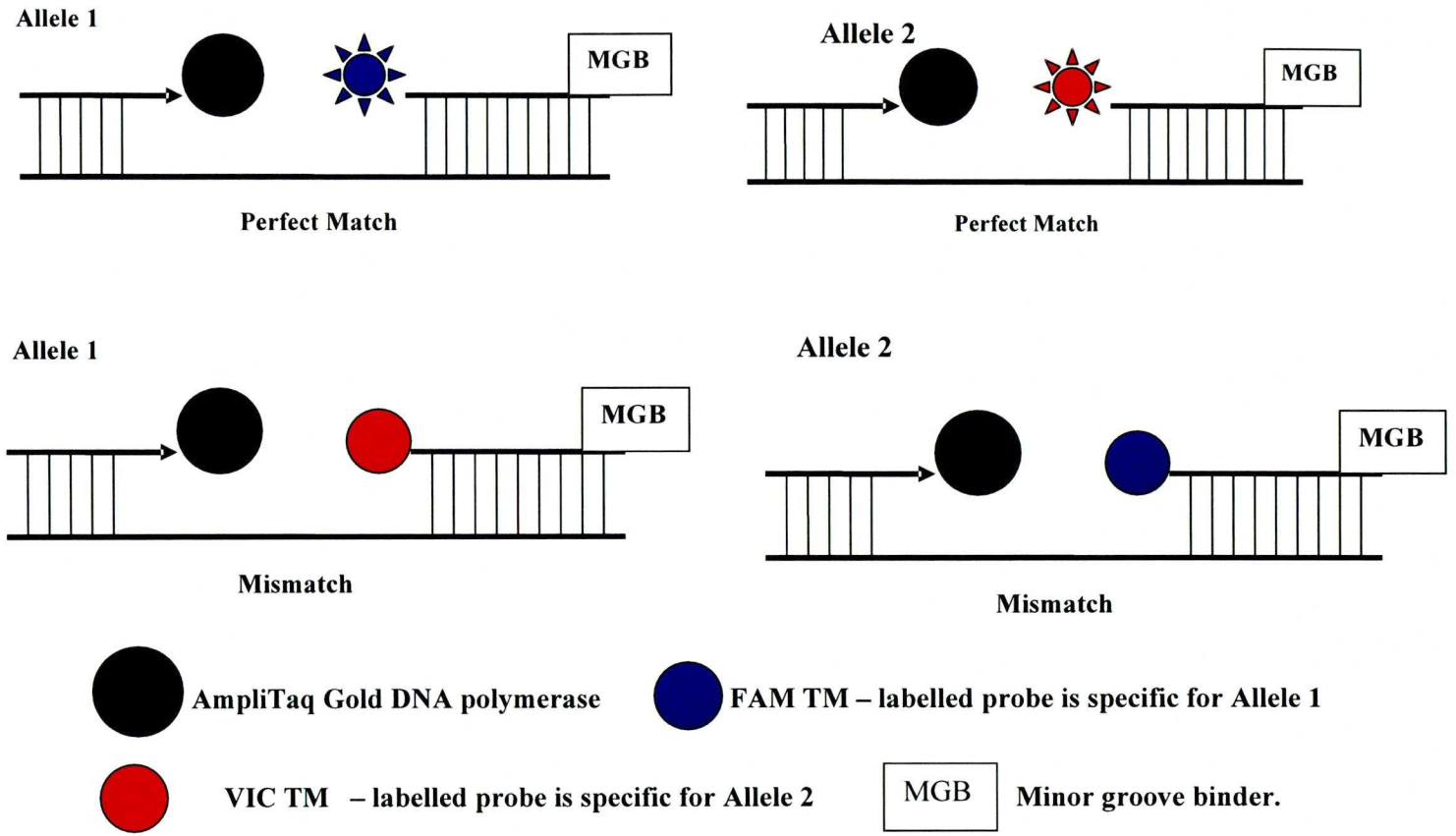


Figure 2.5. Allelic discrimination using Taqman technology

Genotyping was done without knowledge of the GABA-T activity status of the volunteers. All of the reactions were done in the presence of negative controls. 10% of the samples (positive controls) for each SNP were genotyped twice and checked for any discrepancies. These positive controls were randomly selected for each allele of a SNP.

Each 20µl reaction contained 10µl of TaqMan® Universal PCR master mix, 1µl of TaqMan® pre-designed SNP genotyping assay, 1.5µl of DNA and 7.5µl of water. The conditions used for the PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

2.2.7. Genotype- phenotype correlation

GABA-T activity was ascertained in 32 healthy volunteers of Asian and Caucasian origin. These healthy volunteers were genotyped for the 5 selected SNPs in ABAT gene. Their genotype was then correlated with their GABA-T activity.

2.2.8. Statistical analyses

All the statistical tests were performed using SPSS software (SPSS 16.0). Paired Student's t test was used to compare the GABA-T activity of platelets stored for one day and for a week. p value ≤ 0.05 denoted that there was a significant difference in GABA-T activity of platelets under these conditions.

The GABA-T activity in platelets when the assay was done in large volume (2,100 µl) was compared to that when the assay was done on a 96-well plate in a smaller volume (400µl) by unpaired Student's t test. A p value of ≤ 0.05 was considered statistically significant.

One way analysis of variance (ANOVA) was used to compare the means of GABA-T activity at different time points to determine the optimum time required for the first reaction, which is the rate limiting reaction. A p value of ≤ 0.05 implied that there was a significant difference in GABA-T activity at the different time points compared.

The effect of thawing on platelet activity was assessed across the different time points using one way ANOVA. A p value of ≤ 0.05 indicated that there was a statistically significant difference in GABA-T activity in the various groups analysed.

The GABA-T activity of each of the VGB exposed platelet groups was compared to that without VGB treatment using one way ANOVA. Bonferroni correction for multiple testing was applied. A statistically significant difference in GABA- T activity was indicated by a p value ≤ 0.05.

H-W equilibrium principle defines a hypothetical situation in which there is no change in the gene pool (frequencies of alleles), hence no evolution. The allele frequencies were calculated. By using the H-W equilibrium equation, $p^2 + 2pq + q^2 = 1$, where p is the frequency of the dominant allele and q is the frequency of the recessive allele, an expected number of people having a particular genotype (homozygous major, heterozygous or homozygous recessive) can be calculated. The chi-squared test compares the expected and observed counts and states whether there is a significant difference between the two (p value ≤ 0.05).

Genotype-phenotype correlation was undertaken by one-way ANOVA using the SPSS software (SPSS 16.0). Bonferroni correction for multiple testing was done. A p value ≤ 0.05 was taken to be statistically significant.

2.3. Results

2.3.1. GABA-T activity is dependent on platelet isolation method

Platelets were isolated (a) soon after blood collection, (b) from plasma, after allowing the blood to stand for 2hrs, and (c) 2 hrs after adding dextran, and later GABA-T activity was determined. The technique described in section 2.2.4.1 has previously been used to measure the activity of brain GABA-T. For the first time, I used it to measure platelet GABA-T activity. Modifications were made to elucidate the optimum activity of platelet GABA-T activity. There was no significant variation in GABA-T activity in platelets isolated from blood soon after collection and from plasma after 2hrs. Contrast microscopy revealed the platelets were contaminated with RBCs when isolated from blood soon after the collection. In contrast, there was no RBC contamination of platelets when isolated from blood treated with dextran, but a significant decrease ($p \leq 0.05$) in GABA-T activity was observed, as shown in Figure 2.6. No RBCs were found in platelets isolated from the plasma.

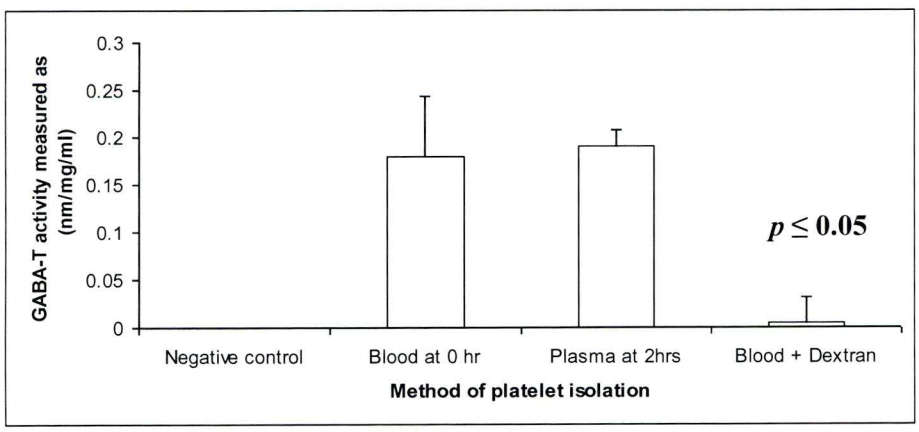


Figure 2.6. GABA-T activity using platelets isolated by different methods. Each experiment was performed 6 times and GABA-T activity was ascertained 6 times in triplicate. There was a significant (ANOVA) decrease in GABA-T activity when platelets were extracted from blood to which dextran was added. The results represent the mean value of 6 experiments with the error bars representing the standard error of the mean (SEM).

2.3.2. GABA-T activity is not affected by storage

The GABA-T activity in platelets was estimated immediately (next day) and after one week of storage at -70°C. There was no significant difference ($p > 0.05$) in GABA-T activity as shown in Figure 2.7.

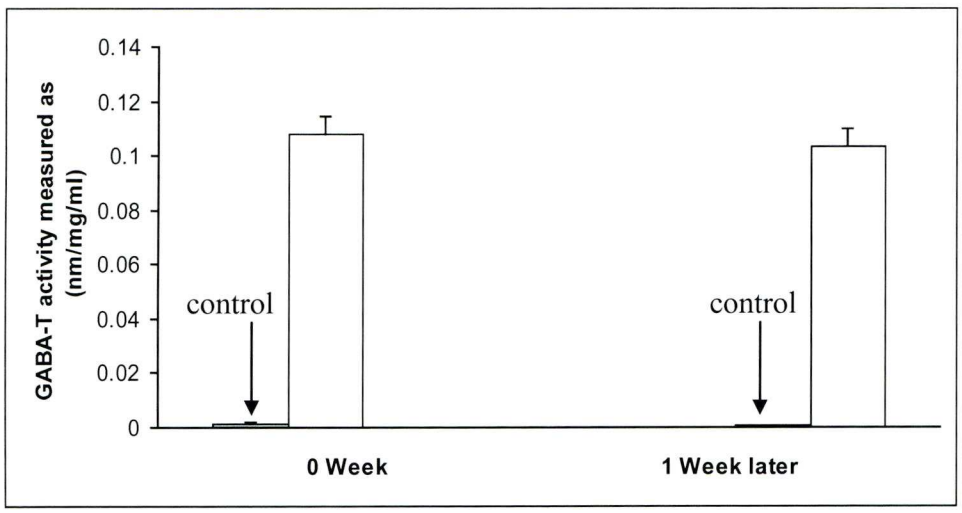


Figure 2.7. Shows the GABA-T activity of platelets isolated the next day (0 week) and after one week. GABA-T activity was ascertained 6 times in triplicates at 0 and 1 week. Results represent the mean value of 6 experiments with the standard error of mean (SEM).

2.3.3. GABA-T activity using a miniaturised assay

No significant difference ($p > 0.05$) in activity of GABA-T was observed when measured using a spectrophotometer and by a Bio-Tek FL600 fluorescence micro plate reader on a 96 well plate, as shown in Figure 2.8.

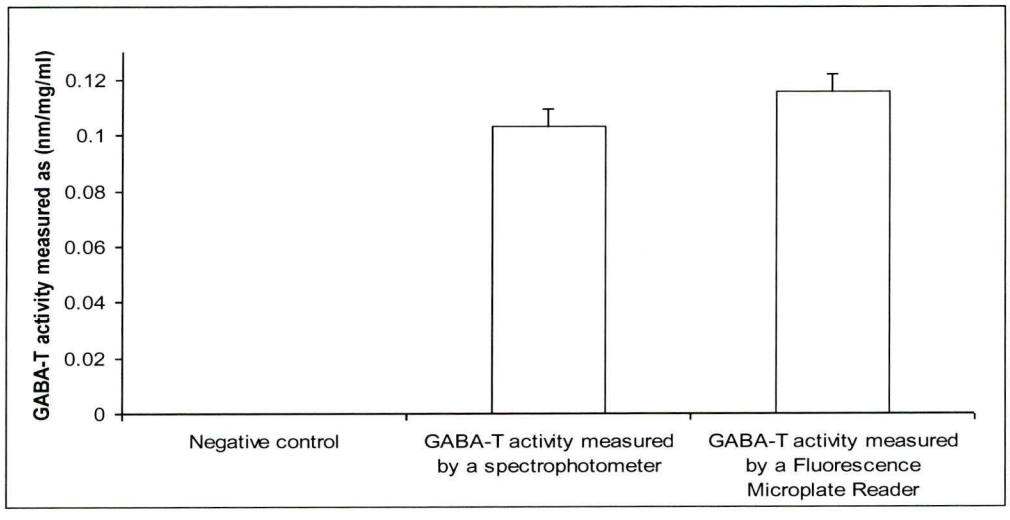


Figure 2.8. Shows the GABA-T activity of platelets read on a 96 well plate. The activity was ascertained 6 times each by a spectrophotometer and by a Bio-Tek FL600 fluorescence micro plate reader at 340nm. Each GABA-T activity assay was done in triplicate. Results represent the mean value of 6 experiments and the error bar represents the standard error of mean (SEM).

2.3.4. Optimum time required for the first reaction.

The first reaction is the rate limiting reaction. There was no significant difference in optical density when the first reaction was stopped at any of the time points assessed (10-90 min), as shown in Figure 2.9. The volume of the first reaction was 100 µl.

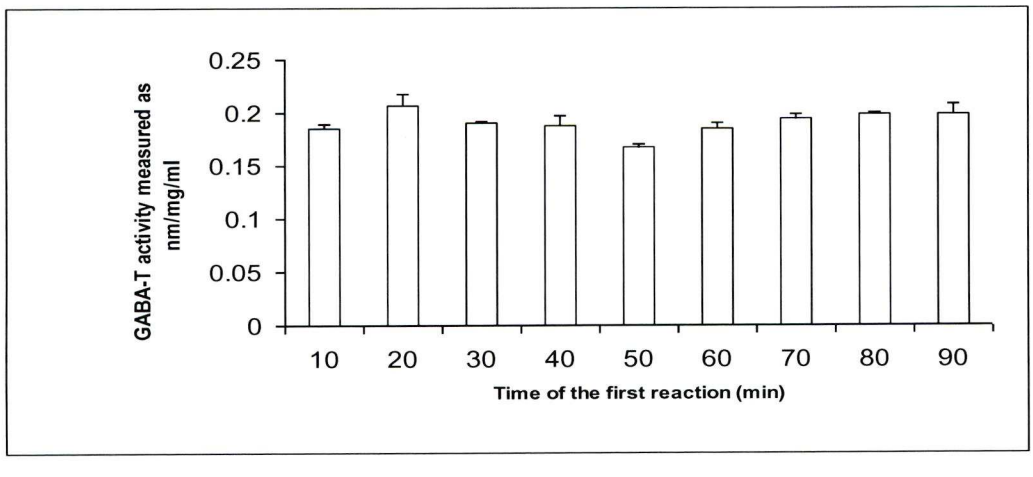


Figure 2.9. shows GABA-T activity when the first reaction was stopped at 10-90min. The experiment was repeated 4 times in triplicates. Results represent the mean value of 4 experiments and the error bar represents the standard error of mean (SEM).

2.3.5. GABA-T activity after removal from the freezer

GABA-T activity was checked 30, 60, 90, 150 min after taking the platelets out of the -70°C freezer. The platelet samples were kept on ice until used for the estimation of GABA-T activity. There was no statistically significant decrease in GABA-T activity until 150 min, as demonstrated in Figure 2.10. There was a downward trend in activity, most pronounced at 150 min, although this did not reach statistical significance.

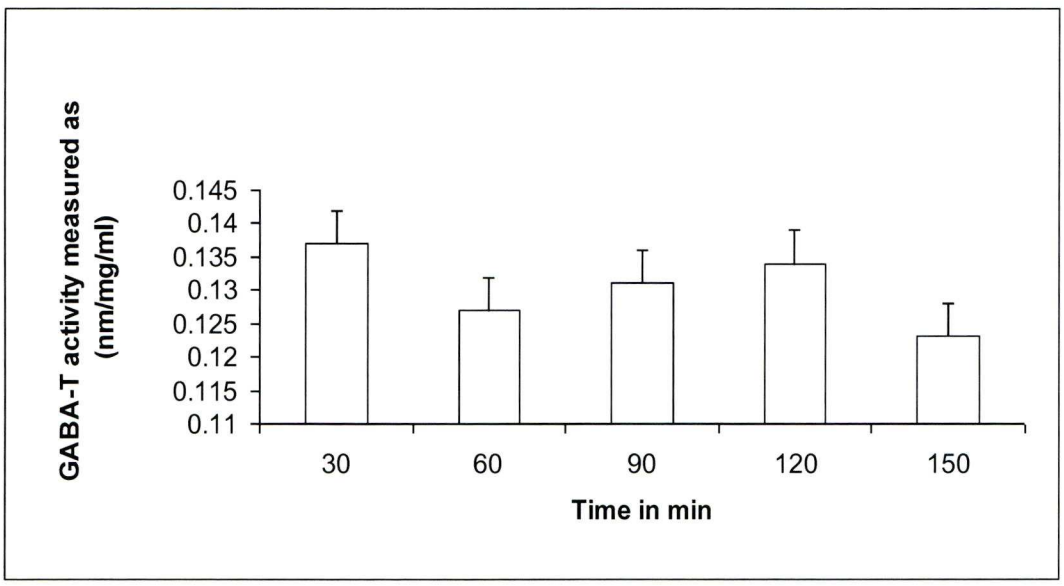


Figure 2.10. Shows GABA-T activity of platelets measured 30-150 min after taking the platelets out of the freezer. This experiment was done 3 times in triplicates. Results represent the mean value of 3 experiments and the error bar represents the standard error of mean (SEM).

2.3.6. Inhibition by vigabatrin (VGB)

Dose-dependent inhibition of platelet GABA-T activity was observed as shown in Figure 2.11. VGB at 3mM concentration produced a statistically significant ($p \leq 0.011$) difference in GABA-T activity when compared to the platelets not treated

with VGB, after correction for multiple comparisons. However complete inhibition of platelet GABA-T activity was not achieved even at 3mM. The GABA-T activity with other concentrations of VGB was not significantly different from that without VGB.

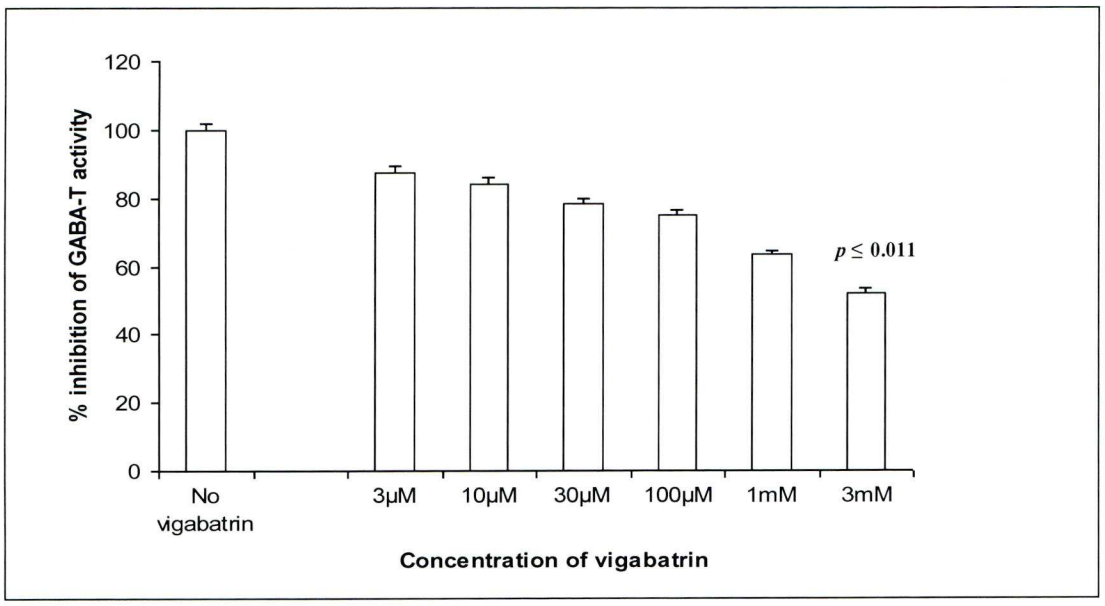


Figure 2.11. Shows inhibition of GABA-T activity by various concentrations of vigabatrin. This experiment was performed six times in triplicates. Results represent the mean value with the standard error of mean (SEM). 3mM of vigabatrin concentration produced a statistically significant (ANOVA, after correction for multiple comparisons) difference in GABA-T activity when compared to the platelets not treated with vigabatrin.

To summarise, the conditions used for GABA-T activity assay for the rest of the experiments were as follows: platelets were isolated and stored for one week before GABA-T activity was estimated. GABA-T activity was ascertained within 30 minutes of taking it out of the -70°C freezer. This assay consisted of 2 reactions. The first one was the rate limiting reaction for 30 min and the second reaction was performed for 90 minutes. The volume of the first reaction and second reactions were

100µl and 300µl, respectively. This assay was read on a 96 well plate by a Bio-Tek FL600 fluorescence micro plate reader at 340nm of absorbance.

2.3.7. Putative functional SNPs predicted in ABAT

Table 2.1 shows the putative functional SNPs predicted in ABAT using the criteria mentioned in the methods section. Out of 745 known SNPs in ABAT (dbSNP), 5 were selected for genotyping. As seen in Figure 2.12, these were present in different locations on the gene.

Figure 2.12. A schematic representation of selected SNPs mapped on to ABAT gene. The blue blocks represent the exons.

Table 2.1. Putative functional SNPs in ABAT to genotype

SNP ID	Position in Chromosome 16	Alleles	Amino acid change	Type	MAF
rs1731017	8747455 (-)	T/C	Q/R	NS-ESE	0.4
rs1641022	8776277 (+)	A/C	V/V	S-ESE	0.439
rs1079348	8780957 (+)	T/C		Intron boundary	0.242
rs1641003	8742317 (+)	C/T		upstream	0.225
rs1345300	8671929 (+)	C/T			0.192

(NS –Non synonymous, S – Synonymous, ESE – Exon splice enhancer, MAF - minor allele frequency).

2.3.8. Allelic frequency of the selected SNPs

Figure 2.13 shows an allelic discrimination plot representative of the genotyping performed. The allelic frequencies of the selected SNPs were ascertained by real time PCR. Table 2.2 below shows the allelic frequencies of the above SNPs in my study population. All the SNPs were in H-W equilibrium (Table 2.3). The allele frequencies were comparable to that in dbSNP.

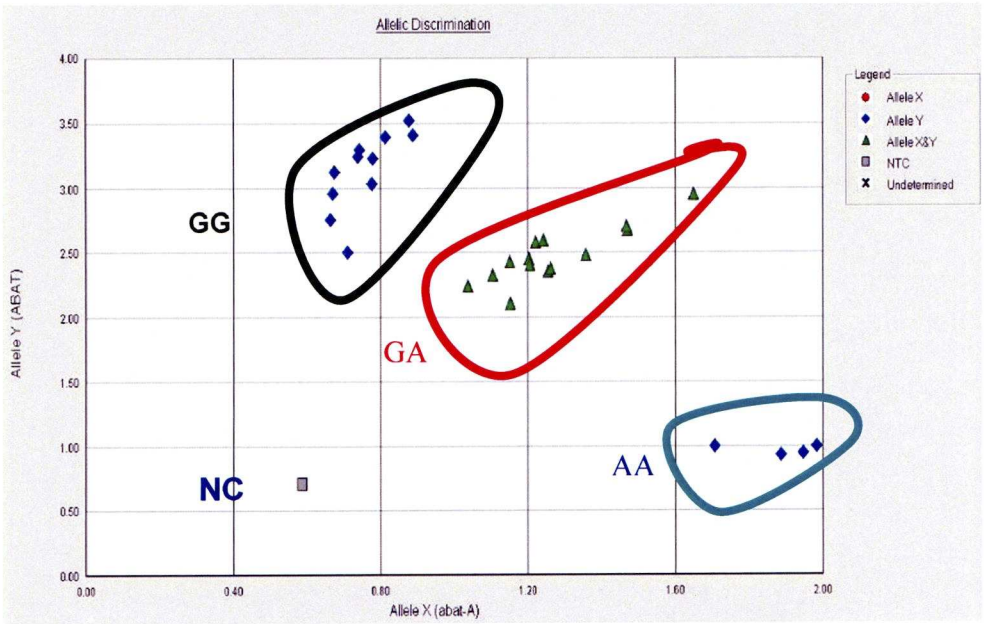


Figure 2.13. An example of allelic discrimination plot of rs1731017 using real time PCR technology by ABI PRISM 7000 Sequence detection system. The homozygous wild type (GG genotype) is represented as blue diamonds, heterozygotes (GA) as green triangles and the homozygous mutant (AA) as blue diamonds. NC is negative control.

Table 2.2. Allelic frequency of genotyped SNPs in ABAT

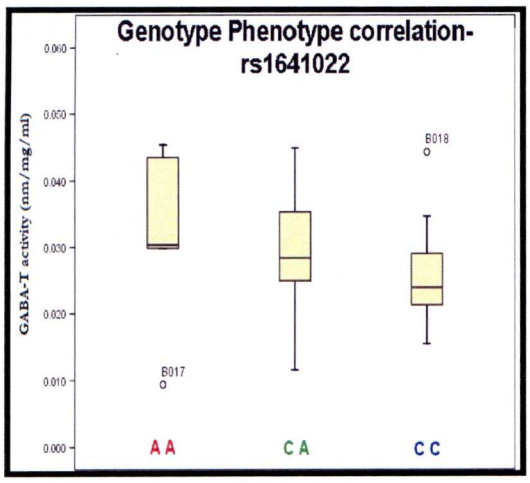
SNP	No. of individuals	Minor allele: frequency	Major allele: frequency	dbSNP Minor allele frequency
rs1731017	139	A: 0.400	G: 0.600	0.400
rs1641022	139	A: 0.439	C: 0.561	0.439
rs1641003	139	C: 0.225	T: 0.775	0.225
rs1079348	138	C: 0.242	T: 0.758	0.242
rs1345300	137	C: 0.192	T: 0.808	0.192

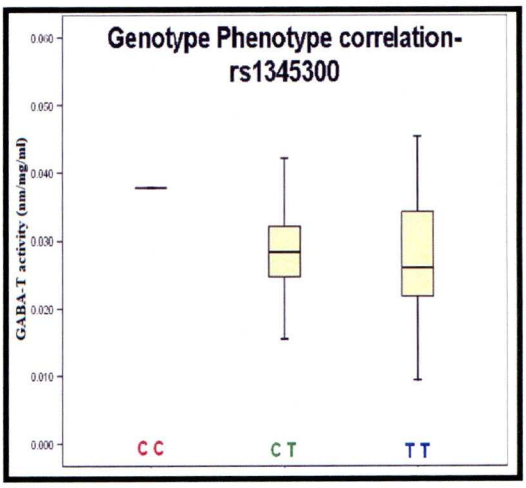
Table 2.3. Demonstrates that the SNPs were in Hardy-Weinberg equilibrium (as shown by the X^2 test)

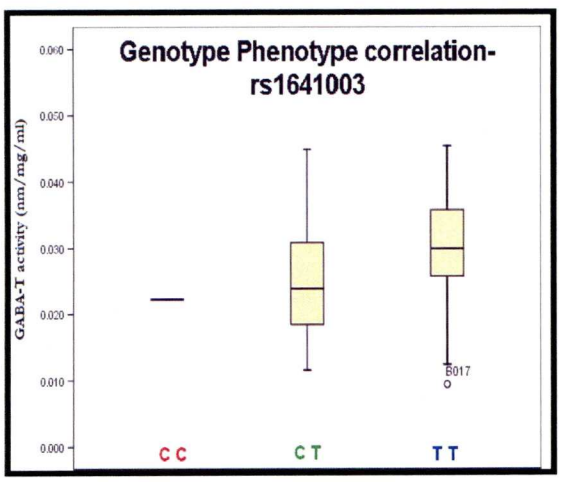
SNP id	Genotype	Observed No. of	Expected No. of peo	X^2	*p* value
	G G	50	50		
rs1731017	G A	67	66	0.004	0.998
	A A	22	22		
	T T	67	79		
rs1079348	T C	59	50	2.906	0.234
	C C	13	8		
	T T	90	89		
rs1345300	T C	39	43	0.893	0.64
	C C	8	5		
	T T	91	82		
rs1641003	T C	43	48	1.128	0.569
	C C	4	6		
	C C	55	43		
rs1641022	C A	64	68	2.359	0.307
	A A	20	26		

2.3.9. Genotype phenotype correlation

The GABA-T activity was estimated in 32 healthy volunteers. The results were expressed as nm/mg/ml protein. This was correlated to the genotype of 5 SNPs. No association was observed between GABA-T activity and SNPs rs1641022, rs1079348, rs1641003 and rs1345300 (Figure 2.14). An association was found between GABA-T activity and SNP rs1731017. The homozygous GG individuals had significantly lower activity than AA (p=0.011) or GA (p= 0.001) individuals as shown in Figure 2.15. rs1731017 is a nonsynonymous SNP leading to a glutamine/arginine (Q/R) amino acid change at position 56 of the GABA-T enzyme. B017 and B018 were outliers (Figure 2.14 and 2.15). Both genotyping and GABA-T activity were estimated three times in these individuals. To the best of my knowledge an association of SNPs and GABA-T activity has not been reported earlier.







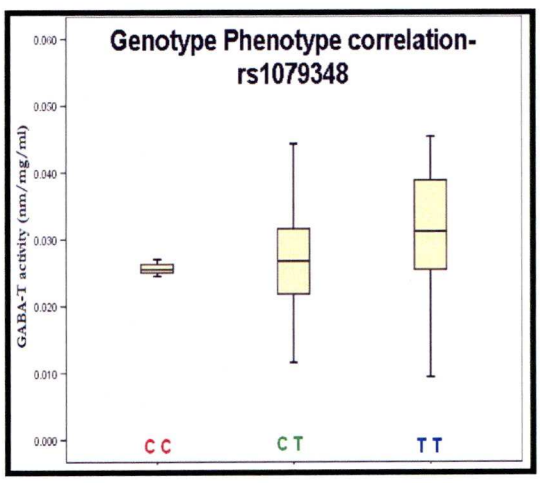


Figure 2.14. Correlation of GABA-T activity in 32 healthy volunteers with different SNP genotypes - rs1641022, rs1345300, rs1641003 and rs1079348. The Y axis represents GABA-T activity and X axis the genotype of healthy volunteers. No association was found between GABA-T activity and any of these SNPs. Individuals B018 and BO17 are outliers.

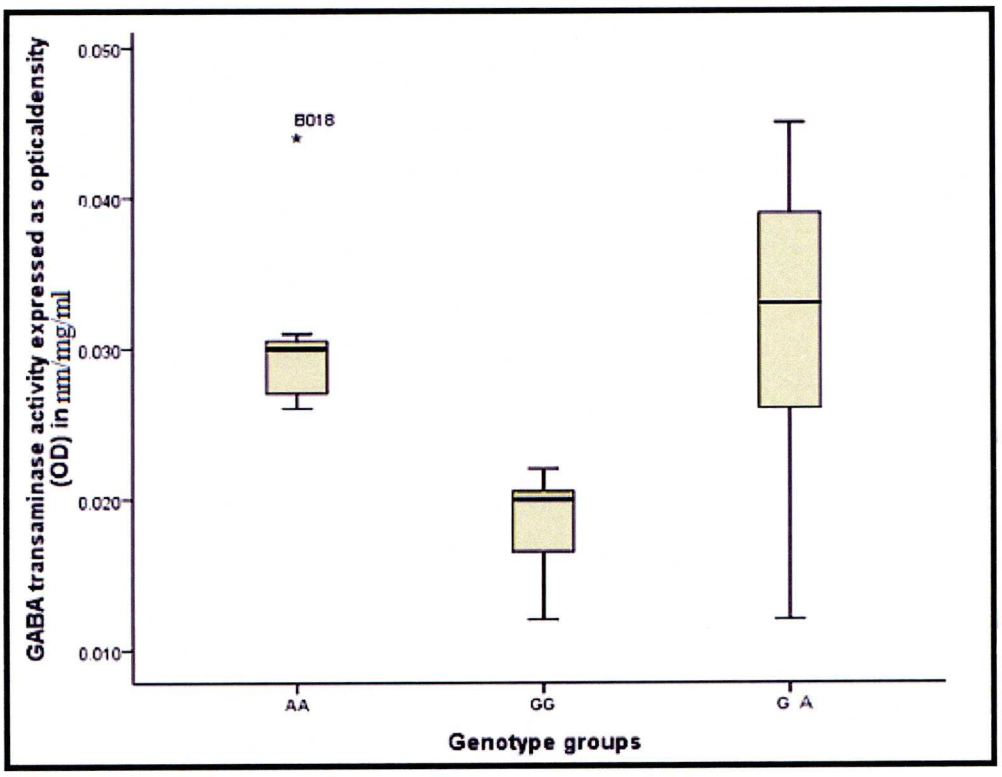


Figure 2.15. GABA-T activity in 32 healthy volunteers with different genotypes for the SNP rs1731017. Y axis represents the GABA-T activity and X axis the genotype of healthy volunteers. GABA-T activity of healthy volunteers with GG was significantly lower than AA & GA. Genotypes AA & GA did not affect GABA-T activity. Individual B018 was an outlier.

2.4. Discussion

Platelets are discoid cells produced by megakaryocytes in the bone marrow. They are released into blood by the fragmentation of the megakaryocyte cytoplasm. Their life span is 9-10 days after which they are replenished (Rodgers, 1999; Camacho and Dimsdale, 2000). Platelets are mainly involved in haemostasis. They have been used as a model for serotonin transport in the brain (Paasonen, 1968; Lingjaerde, 1969). They also contain uptake mechanisms for many amino acid transmitters including GABA, glutamate, aspartate and glycine, similar to those present in the CNS (Zieve and Solomon, 1968; Mangano and Schwarcz, 1981; Hambley and Johnston, 1985). Indeed, platelets have been used to explore the etiopathogenesis of many neuropsychiatric diseases (Mangano and Schwarcz, 1982; Ferrarese, et al., 1999; Ferrarese, et al., 2001a; Ferrarese, et al., 2001b; Ferrarese, et al., 2001c). The characteristics of platelet GABA-T are similar to those of brain GABA-T. The kinetic, molecular and pharmacological properties of these enzymes are comparable. They are both inhibited by VGB and other inhibitors and they have the same substrates. However, platelet GABA-T activity is much lower than that of the brain GABA-T (White, 1979; White and Faison, 1980; Sherif, 1994). It has been proposed that platelet GABA-T activity can be used as a model of brain GABA-T activity (Arteaga, et al., 1993; Sherif, 1994). For this reason, I used platelets as a model system to assess GABA-T activity.

I have described the standardization of a GABA-T assay in platelets. This is a spectrophotometric method, hence not as sensitive as radiometric assays or gas chromatography–mass spectrometry (GC-MS). This assay is a coupled reaction in that it involves two reactions and the product of one reaction is the substrate of the

second reaction. This assay thus measures the GABA-T activity indirectly. As shown in Figure 2.3, this assay measures the NADH formed not succinic semialdehyde. In my experiments, the GABA-T activity did not significantly decrease over time. This could be due to the fact that the rate of formation of NADH may not be equal to the rate of formation of succinic semialdehyde. There could be a lag time for the formation of NADH. Also coupled reactions can give higher values when the enzyme concentration is less as in the case of estimation of creatinine kinase activity measured by coupled reaction (Dinovo, et al., 1973). Also there was no significant difference in GABA-T activity when the time of the first reaction, which is the rate limiting reaction was changed from 10-90 min. In a coupled reaction, for example in the reaction in Figure 2.3, if the measurement of NADH has to be a direct measure of the GABA-T activity, the velocity of glutamate dehydrogenase enzyme should approach infinity and the reaction should be irreversible (Dinovo, et al., 1973). This is hardly the case in most of the reactions conducted in the laboratory. Time is required to achieve steady state concentration of the intermediate products; thus the acetylpyridine NADH formed may be less than succinic semialdehyde formed. The second reaction is a reversible reaction, further reducing the concentration of acetylpyridine NADH.

There are no in vitro studies exploring the effect of VGB on human platelets. However, the IC50 values of VGB for neuronal GABA-T vary from 89- 200μM (Gram, et al., 1989). I used concentrations between 3μM – 3mM of VGB to inhibit platelet GABA-T. This concentration is well above the concentration of VGB achieved in healthy volunteers taking 1 g, 2 g and 4 g doses of VGB. At these doses of VGB, the GABA-T activity was 43%, 30% and 21%, respectively compared with

the mean control value (Rimmer, et al., 1988). VGB, a specific inhibitor of GABA-T however was not able to totally abolish platelet GABA-T activity in the assay reported here. While other studies (Rimmer, et al., 1988; Arteaga, et al., 1992) have been able to reduce the platelet GABA-T activity by around 70-75%, my experiments could only decrease this by 50%. This could be due to the above mentioned reasons or it may suggest that the GABA-T assay is not specific. In the assay described, I have measured the amount of acetylpyridine NADH formed (Figure 2.3). Platelets contain NADH as a part of their redox regulation in platelet adhesion (Seno, et al., 2001; Arthur, et al., 2008), and the assay may also be measuring this NADH. It is known that platelets contain GABA, glutamic acid and glutamate dehydrogenase (Oset-Gasque, et al., 1986). So even before the start of the assay, acetyl pyridine NADH could be formed, which is also measured by the assay. Additionally platelets were treated with VGB for only 10 min – it is possible that greater GABA-T inhibition could have been achieved with a longer incubation. Despite these possible limitations, VGB did inhibit GABA- T activity in this assay suggesting that GABA – T is definitely present in platelets. All platelet samples from healthy volunteers were treated similarly. Therefore, though not an ideal assay, conclusions can be drawn from these experiments.

Platelet isolation requires 3-4 hrs. Ascertaining the GABA-T activity takes another 3-4 hrs. Usually the GABA-T activity of platelets from 6-8 individuals is assessed at the same time. Hence platelet isolation and estimating the GABA-T activity on the same day is not economical in terms of time, effort and resources. Therefore, I checked the GABA-T activity of platelet samples after storing at -70°C for a week. As shown in Figure 2.5, the GABA-T activity was maintained after a week of storage.

Next I scaled down the reaction volumes, since it is cost effective and less time consuming to perform the GABA-T assay. This reduction did not affect the GABA-T activity as seen in Figure 2.6. Potassium phosphate buffer was used instead of the Tris-HCL used by Schousboe et al. (1973). I used phosphate buffer since Tris buffers inhibit GABA-T activity. Tris forms a complex with pyridoxal phosphate, the cofactor essential for the functioning of GABA-T. This reduces the amount of pyridoxal phosphate available for the activity of GABA-T (White and Sato, 1978). The GABA-T activity in platelets varies considerably across species (Sherif, et al., 1993). As mentioned earlier, GABA-T enzyme is widely distributed in the CNS and other body tissues. GABA-T activity is higher in the grey matter than in the white matter (Sherif, et al., 1992a). This enzyme is present in both neurons and the glia. However most of its activity is in the neurons (Bedoya, et al., 1988; Larsson and Schousboe, 1990). In rats, an increase in GABA-T activity was reported with age and sex. Male rats showed 15-25% higher activity than females (Aoyagi, et al., 1990; Sherif, et al., 1991). In humans, no age or sex related changes in brain or platelet GABA-T activity have been reported (Berrettini, et al., 1982; Armijo, et al., 1989; Arteaga, et al., 1993; Sherif, et al., 1992b). There is no seasonal or diurnal variation in GABA-T activity in a single individual, though great inter-individual variability exists (Rimmer, et al., 1988; Bolton, et al., 1989). It has also been shown that GABA-T activity decreased significantly when platelets were isolated after a meal (Berrettini, et al., 1982). Therefore, I used fasting blood for platelet isolation to determine the GABA-T activity in healthy volunteers.

Kinirons et al. (2006a) used a tagging method to identify putative functional SNPs in the ABAT gene. I used the criteria mentioned above (2.2.5) to identify putative functional SNPs in the ABAT gene. All the SNPs genotyped were in H-W

equilibrium. These SNPs included SNPs in the 5' UTR region, coding and intronic regions (Table 2.1). The synonymous SNP rs1641022 is close to the active site at position 329 while rs1641022 and rs1731017 are predicted to be exon splicing enhancers (ESE) according to the software programs I used in selecting the SNPs. ESE sequences are present in the exons and they are involved in constitutive and regulated splicing (Blencowe, 2000). Variation in these sequences could lead to exon skipping and defective alternate splicing. SNPs in the intronic regions could also affect RNA splicing (Brockmöller and Tzvetkov, 2008). All these SNPs were in highly conserved regions too.

There are no studies to date, which have looked into the association between GABA-T activity and SNPs in the ABAT gene. I looked at the correlation between GABA-T activity and selected SNPs in 32 healthy volunteers. Studies have shown GABA-T activity varies in different types of epilepsy (Arteaga, et al., 1993; Rainesalo, et al., 2003). Twin studies have demonstrated that GABA-T activity may be determined by genetic factors (Berrettini, et al., 1982). It has been proposed that genetic factors play a major role in determining GABA-T activity (Berrettini, et al., 1982; Rainesalo, et al., 2003) . For the first time, I showed an association between GABA-T activity and SNP rs1731017. VGB acts by inhibiting the GABA-T enzyme. SNP rs1731017 is a non synonymous SNP which results in a Q/R amino acid change in the GABA-T enzyme. Though this SNP is not near to any of the active sites in the primary structure, the SNP is not far from the active site in its tertiary structure as seen in Figure 2.16. This could potentially affect the activity of GABA-T.

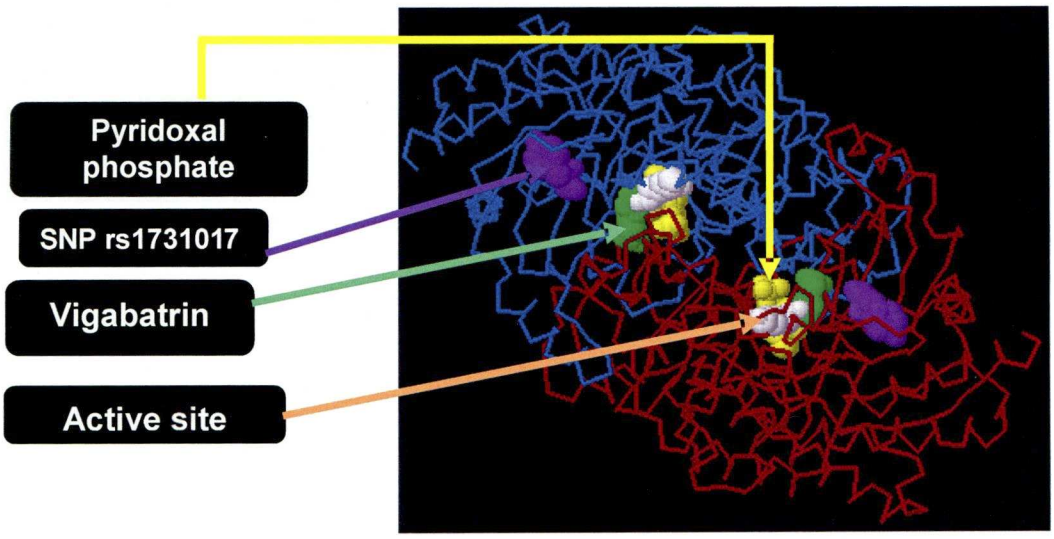


Figure 2.16. Showing the SNP rs1731017 in GABA transaminase enzyme. Only one subunit of this enzyme is shown which consists of 2 identical monomers represented in blue and red colours.

In my study population, according to H-W equilibrium equation ($p2+q2+2pq = 1$), the minor allele frequency should be at least 0.4 to have five individuals who are homozygous for the minor allele. Then I could look at the association between homozygous minor allele, homozygous major allele, heterozygous, and the GABA-T activity. Among the SNPs selected, only rs1731017 and rs1641022 had a minor allele frequency above 0.4. To detect association between the other SNPs and GABA-T activity a larger study population is required. Therefore, I cannot conclusively say that there is no association between other SNPs genotyped and GABA-T activity.

Clearly, there is a necessity to look at variants affecting genes, which are involved in the pharmacokinetics and pharmacodynamics of VGB in a large patient population. This could help us identify SNPs which predispose to VVFD, and help us develop a

screening test to prescribe this highly effective AED in selected patients (Coppola, et al., 1997; Mitchell and Shah, 2002), who will benefit from this drug, especially the paediatric patients with infantile spasms. I have established that variation at SNP rs1731017 affects the activity of GABA-T. The next step will be to investigate GABA-T activity in VVFD patients and compare it to that of patients who have normal fields in spite of being treated with VGB.

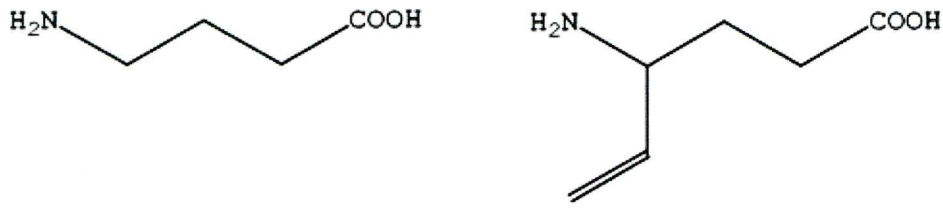
Chapter 3

Estimation of GABA-T activity in patients with vigabatrin-induced visual field defects

3.1. Introduction

VGB, an antiepileptic drug, with a similar structure to GABA (Figure 3.1) has been used in the treatment of complex partial seizures (Coppola, et al., 1997; Cramer, et al., 1999; Satishchandra, et al., 1999; Coppola, 2004) and infantile spasms (Aicardi, et al., 1996; Appleton, et al., 1999; Mitchell and Shah, 2002) since 1989 in more than 60 countries (Hisama, et al., 2001). However Eke et al. (1997) reported three patients who developed peripheral visual field loss after taking VGB (Figure 3.3). Figure 3.2 shows the normal isopter positions in the peripheral visual field (Niederhauser and Mojon, 2000). When compared to Figure 3.2, all the patients had loss of peripheral visual field (Figure 3.3). Their central visual field was intact. All three patients had tunnel vision. Their vision and the peripheral visual field constriction did not improve after stopping VGB.

3.1a **3.1b**



GABA **Vigabatrin**

Figure 3.1. Showing the structures of GABA (3.1a) and Vigabatrin (3.1b)

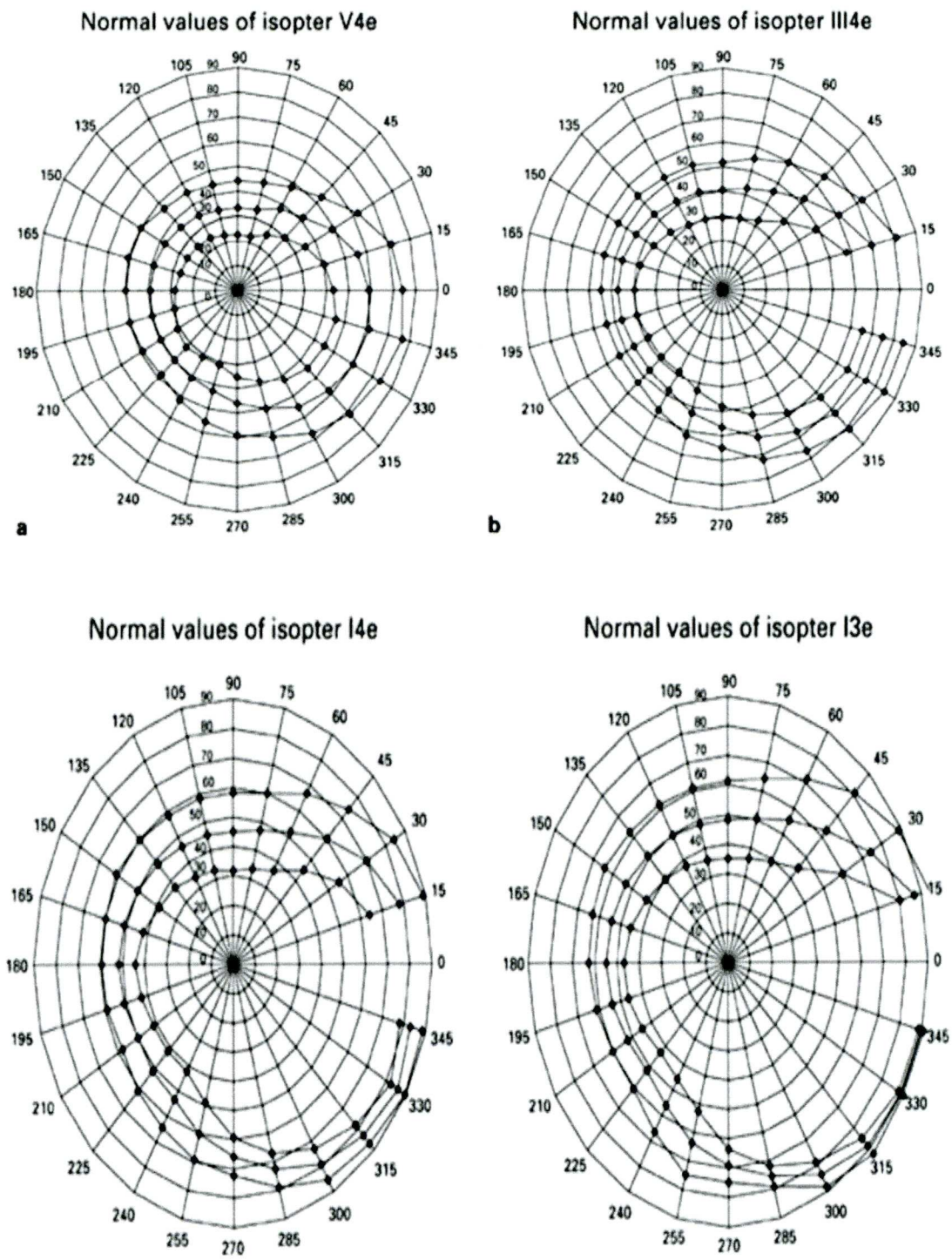


Figure 3.2. Normal positions of the isopters. The average positions ± 2 standard deviations are plotted. (Niederhauser, et al., 2000)

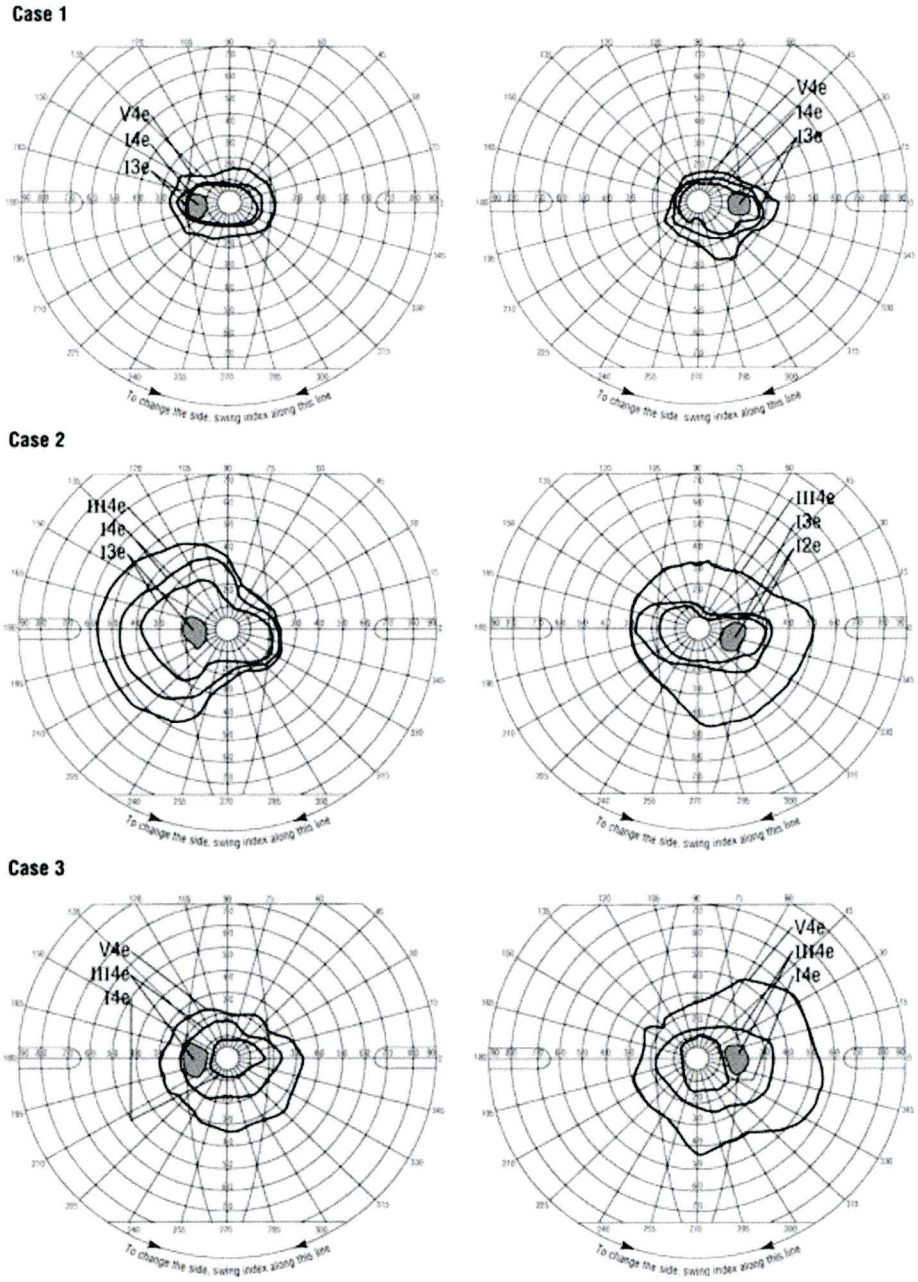


Figure 3.3. Goldmann visual fields 37 months after start of vigabatrin treatment (case 1, top), 28 months after start of vigabatrin treatment (case 2, centre), 38 months after start of vigabatrin treatment (case 3, bottom) (Eke et al. 1997).

VGB causes VFD in approximately 30-50% of patients. The VVFD consists of bilateral constriction of the peripheral visual field. The nasal field is usually extensively affected (Wild, et al., 1999; Lawden, et al., 1999; Gross-Tsur, et al., 2000; Gross-Tsur, et al., 2002). The severity can be mild to severe; however VFD does not affect the routine activities of the majority of patients. There are also reports of mild to moderate involvement of the central retina (Krauss, et al., 1998; Nousiainen, et al., 2000b; Manuchehri, et al., 2000). They include colour vision disturbances, and reductions in contrast sensitivity and visual acuity (Eke, et al., 1997; Krauss, et al., 1998; Nousiainen, et al., 2000a; Nousiainen, et al., 2000b). Electrophysiological studies have also demonstrated defects in both the outer and inner retina at the level of the Müller cells (Coupland, et al., 2001). VVFD has unique electroretinogram findings (Nousiainen, et al., 2000a). Males have a two-fold greater risk of developing VVFD (Wild, et al., 1999; Hardus, et al., 2000a; Hardus, et al., 2000b). Evidence for the association between VVFD and duration of treatment and dose of VGB has been contradictory (Wild, et al., 1999; Lawden, et al., 1999; Manuchehri, et al., 2000; Newman, et al., 2002; Kinirons, et al., 2006b). However, a recent large prospective cohort study reported that male gender, duration and dose of VGB are risk factors for the development of VVFD (Wild, et al., 2007). VVFD has an insidious onset. The earliest period at which VVFD has been documented in complex partial seizures is 11 months. VVFD appears on an average after 5.5-8 yrs treatment with VGB (Ovation Pharmaceuticals, 2007).

The exact aetiology of VVFD is not known. Possible explanations include direct toxicity of GABA (Hosking and Hilton, 2002) or VGB (Sills, et al., 2001) to the retina, ischaemia in the retina caused by GABA (Hosking and Hilton, 2002), abnormal visual fields associated with complex partial seizures (Hisama, et al.,

2001) and an idiosyncratic reaction to VGB (Newman, et al. 2002; Best, et al., 2004; Kinirons, et al., 2006; Wheless, et al., 2007). Although the mechanism of development of VVFD is not known, the site of injury appears to be the retinal ganglion cells (Best and Acheson, 2004; Wheless, et al., 2007).

In chapter 2, I described the validation of a platelet GABA-T assay. In initial studies, this assay was used in healthy volunteers with known common polymorphisms. However, it is important to note that investigation of healthy volunteers only allows an assessment of common polymorphisms, and one cannot rule out the presence of rare mutations in the ABAT gene itself or in other genes which may control its activity. In view of this, the purpose of this chapter was to investigate the activity of GABA-T in platelets taken from patients who had been prescribed VGB and (1) who developed VVFD and compare them to (2) patients without the visual field defects. In effect, although I had previously utilised a genotype to phenotype approach, in this chapter, I reverted to the conventional paradigm of using a phenotypic assay to identify differences in the activity of a candidate enzyme.

The aim of this chapter was:

> To estimate and compare GABA-T activity in patients who developed VVFD and in patients who did not develop VVFD.

3.2. Methods

3.2.1. Materials

Optiprep was purchased from Axon Lab AG, Le Mont-sur-Lausanne, Switzerland. All the other chemicals used for the experiments mentioned in this chapter were purchased from Sigma Aldrich, Poole, England.

3.2.2. Subjects

This study was a sub-study of the multicentre study – "Pharmacogenetics of GABA-ergic mechanisms of benefit and harm in epilepsy: a retrospective study of genetic and environmental factors determining potential harm from vigabatrin therapy". This was a collaborative project by Charing Cross Hospital, the National Hospital for Neurology and Neurosurgery, the Sanger Institute and the University of Liverpool. The patients for this study were selected from the patients recruited from the above mentioned main study. Patients over the age of 12 with any type of epilepsy, who took VGB for at least 12 months, were included in this study. Patients who are blind or partially sighted (non-VGB aetiology) or having significant learning difficulties were excluded from this study. Fresh blood was required for this study. Therefore, only patients from Walton Centre for Neurology and Neurosurgery, Liverpool were recruited. Out of 358 patients recruited for the main study only the first 14 who agreed to participate in the study were recruited, since this was a pilot study. This could introduce selection bias of patients. However, the mean age and duration of VGB therapy of these 14 patients (51.57yrs, 5.39 respectively) did not significantly differ from the patients in the main study (49.12 yrs, 6.01 yrs, unpublished data). 93.3 % of patients had partial seizures similar to this pilot study (92.8%).

Four experts looked at the visual fields (this comprised of three ophthalmologists and one vision scientist). Visual fields were assessed by Humphrey Field Analyzer, by three consultant neuro-opthalmologists separately who were blinded to all clinical information. A database manager entered their diagnosis in a database. If there was a disagreement on the results, the issue was discussed with the fourth person acting as chairperson until the problem is resolved (i.e. they agree upon one definition). Patients were assigned to the following categories by neuro-ophthalmologists:

- Normal
- VGB associated defect: Either binasal or generalised field constriction with normal fundoscopic examination
- Abnormal non VGB related: Hemianopic/ Central field defects

  Unreliable: Patients who exhibit responses greater than the standard accepted criteria for normality (>20% fixation losses, >33% false negative responses, >33% false positive responses) on visual field assessments.
- Inconclusive: Patients whose fields manifest so high a degree of threshold variability within test and between tests to render accurate interpretation of the field impossible.

Power calculations were not done, as this was a pilot study. Only 14 patients from Walton Centre for Neurology and Neurosurgery, Liverpool agreed to enter this pilot study. These 14 patients with epilepsy were treated with VGB. None of the patients was on VGB at the time of recruitment, even those who did not have any evidence of VVFD. Seven of the patients had VGB associated defect and seven of them had normal visual fields. University College London/ University College London hospital Committees on the Ethics of Human Research (Committee A) approval was

obtained for this study and a written consent was obtained from all the patients recruited for this study.

The demographic characteristics of the patients in this study are given below (Table 3.1). Fourteen patients who had been treated with VGB were recruited for this study. The duration of treatment of VGB was longer in patients who developed VVFD but this was not significantly different from those patients without the VVFD (Table 3.1). The ages of the patients in the two groups were similar, but there was discordance in the gender ratios between the two groups, although this was not statistically significant because of the small numbers studied.

Table 3.1. Showing the demographic characteristics of patients recruited for this study

	Visual field not affected	**Visual field affected**	***p* value**
Number of Males	1	4	**0.094**
Number of females	6	3	
Age	56 ± 11.045	47.14 ± 9.616	0.136
Seizure type			
Partial	6	7	0.299
General	1	0	
Duration of vigabatrin therapy			
Mean (yrs)	3.85 ± .95	6.93 ± 1.25	**0.072**
Range (yrs)	1.17-6.17	1.67-11.17	

3.2.3. GABA-T activity

Blood samples were collected from patients 2hrs after food. Platelets were isolated as mentioned above. GABA-T activity of the platelet samples was estimated, as

described in chapter 2. Bradford method (Bradford, 1976) was used to estimate the protein concentration (see chapter 2). GABA-T activity was expressed as nm/mg/ml.

GABA-T activity values decreases significantly after food (Berrettini, et al., 1982). Because of logistical difficulties, it was not possible to get fasting blood samples from patients. Therefore, initial experiments were conducted in one healthy volunteer to determine the effect of food on GABA-T activity. A fasting blood sample and a blood sample after 2hrs of food consumption were collected from the healthy volunteer. Plasma was separated and platelets isolated, and GABA-T activity measured as described above and in chapter 2.

3.2.4. Statistical analyses

The Chi-squared test (for comparisons of the categorical data) and Student's t-test (for comparisons of continuous data) were used to compare the distributions of demographic characteristics among patients with and without VVFD. The categorical data were sex and epilepsy syndrome, and the continuous data were age and duration of treatment. A p value of ≤ 0.05 was considered significant.

3.3. Results

3.3.1. GABA-T activity

It is reported that food affects GABA-T activity (Berrettini, et al., 1982). It was not possible to collect fasting blood samples from patients. Therefore, GABA-T activity was estimated in fasting blood samples and in 2 hr postprandial blood samples from one healthy volunteer. There was no significant difference in GABA-T activity between the fasting samples and those taken after 2 hours of food intake (Figure 3.4), in the healthy volunteer.

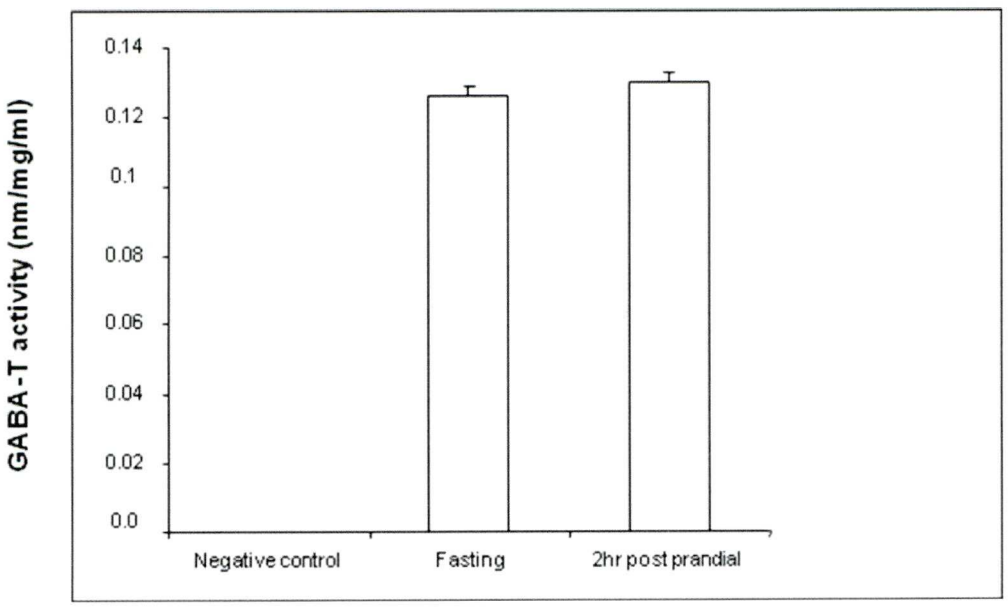


Figure 3.4. GABA-T activity(expressed as nm/mg/ml) in platelets on the Y-axis, using blood from one fasting healthy volunteer and blood withdrawn 2 hrs after food intake. This experiment was done 3 times in triplicates on one individual. Results represent the mean value with the error bars representing the standard error of mean (SEM).

3.3.2. GABA-T activity in patients

There was a significant difference (p = 0.002) in GABA-T activity between patients with and without VVFD (Figures 3.5 and 3.6). The mean GABA-T activity in patients with VVFD was 0.012 ± 0.003 nm/mg/ml compared to 0.025 ± 0.002 nm/mg/ml in those without the VVFD.

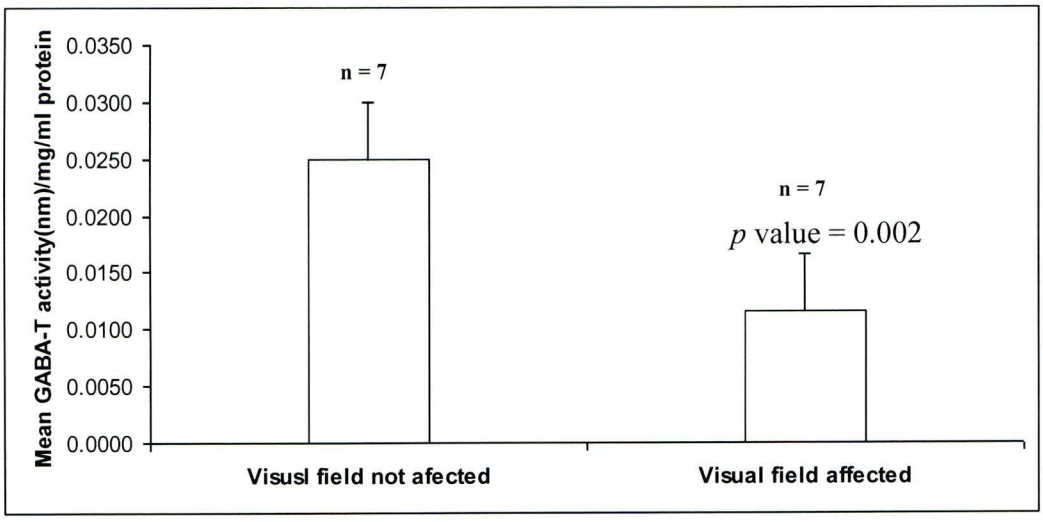


Figure 3.5. GABA-T activity (nm/mg/ml) in patients with and without vigabatrin-induced visual field defects. GABA-T activity in each sample was estimated at least 3 times in triplicate and the mean value was used. The error bars denote the standard error of mean (SEM). Statistical analysis was performed by Student's t test.

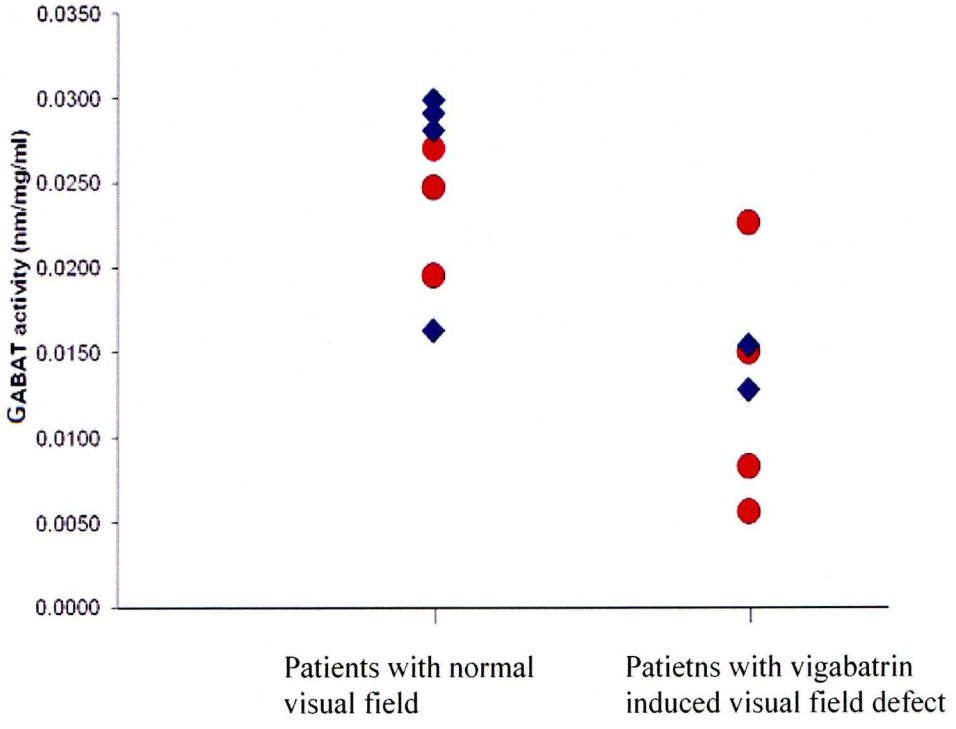


Figure 3.6. The values of individual patients are shown in the scatter diagram. Although there was some overlap in the individuals, a clear distinction can be seen between patients with and without VVFD. Also indicated on the figure are those patients who are males (red circles) and those who are females (blue diamonds). Within the group with VVFD, there was no difference in activity between males and females (mean values for males and females were 0.120±0.01 and 0.113±0.01 nm/mg/ml respectively).

3.4. Discussion

This study has demonstrated a statistically significant difference (p value = 0.002) in GABA-T activity between patients with VVFD when compared to patients without VVFD, with the GABA-T activity being significantly lower in patients with visual field defects. To the best of our knowledge, this is the first time this has been demonstrated in patients with VVFD. I used the method described in chapter 2 to

estimate GABA-T activity, which is not the ideal method to estimate GABA-T activity, as mentioned in chapter 2. Nevertheless, since all the samples were treated uniformly, I could still draw some conclusions that have to be repeated in a larger trial, preferably a prospective trial.

Although the duration of VGB treatment in patients who developed VVFD was longer than in those who did not develop VVFD, this was not statistically significant (p value = 0.072). Many studies, including a recent prospective study by Wild et al. (2007), have shown that VVFD is seen in patients who are treated with VGB for an average of 5.5-8 yrs. The lack of a statistically significant correlation between VVFD and the duration of treatment in this study is probably due to the small sample size. The crucial issue is whether those individuals without VVFD, i.e. controls, might have gone on to develop the visual field deficit. Clearly it is impossible to be sure about this given the retrospective nature of the study, and our data does need to be replicated in another cohort. A number of studies including Wild et al. (2007) have reported that VVFD is more prevalent in males than in females. In our study, I had more males in the group with VVFD; this was again related to the availability of patients locally, and ideally it would have been better if the patients were truly sex-matched. This is something that needs to be tackled in a replication cohort, which unfortunately I could not do because of time constraints. Nevertheless, it is important to note that there was no difference in the GABA-T activity in males and females with VVFD as demonstrated in Figure 3.6.

Previous studies have shown that GABA-T activity decreases after food (Berrettini, et al., 1982). However, it was difficult for us to be able to test the patients while they were fasting, and I therefore estimated the platelet GABA-T activity 2 hours

after food. I did not find a significant difference in GABA-T activity at this time when compared to the GABA-T activity estimated in platelets from fasting blood. This contradicts the findings of Berrettini et al. (1982); the reasons for this are unknown but may have been due to differences in assay sensitivity or dietary factors. It is important to note that other studies which have explored platelet GABA-T activity in epilepsy patients did not use fasting blood samples (Arteaga, et al., 1993; Kumlien, et al., 1995; Rainesalo, et al., 2003).

Our finding that GABA-T activity is significantly reduced in VVFD patients when compared to patients with normal visual fields is novel and highly interesting. GABA-T activity has been evaluated in epilepsy (Sherif and Ahmed, 1995). In our study there was no significant difference in the epilepsy type among patients with VVFD and those without VVFD. The GABA-T activity of the patients in our study was lower than healthy volunteers estimated in chapter 2. Some of the studies have also reported lower GABA-T activity in patients with epilepsy (Rimmer, et al., 1988) when compared to healthy controls. Some have reported higher GABA-T activity in patients with partial epilepsy (Arteaga, et al., 1993; Kumlien, et al., 1995) when compared to healthy controls. However, other studies found no significant difference in GABA-T activity between partial epilepsy patients and healthy volunteers (Rainesalo, et al., 2003). Sex, age and seizure frequency did not affect GABA-T activity. Generalised epilepsy syndromes have a predisposition to occur in families (Kinirons, et al., 2008). That is, there is a genetic predisposition. GABA-T activity in monozygotic twins also shows a strong correlation (Berrettini, et al., 1982). Thus genetic factors are known to affect GABA-T activity, and the inter-patient variability observed in this study that might be due to this. All our patients

with and without VVFD except one, had partial epilepsy and were thus well matched.

The demonstration of a reduction in GABA-T activity in patients with VVFD has to be contrasted with the lack of correlation between most of the SNPs that were studied in chapter 2 (except one). However, the SNP (rs1731017) which was functionally active has not been shown to be a predisposing factor for VVFD (Kinirons, et al., 2006a). Kinirons et al. (2006a) measured the visual field of patients using the Goldmann test, which is a manual test. Since this is a subjective test, even with utmost care to avoid unreliable visual fields, variation in the measurement of visual fields can happen. Ross et al. (1984) reported a 14% variation in repeated visual field testing in individuals with normal visual fields. The visual field of the patients in this study was assessed by Humphrey Field Analyzer, where the test is computerized. However, this too has a subjective nature which can lead to variable test results.

Clearly the approach taken by the above study (Kinirons, et al., 2006a), and in chapter 2, was to investigate common genomic variants (minor allele frequency more than 5%). From the data presented, it seems unlikely that common variants in the ABAT gene predispose to VVFD unless the effect size is very small. Given our finding of a difference in GABA-T activity between patients with and without VVFD, I cannot exclude the possibility that more rare variants, either individually or in combination, predispose to VVFD. This hypothesis can only be examined by direct sequencing of the gene, which is on-going at present. In effect, although the prevailing hypothesis in association studies has been one that common variants

cause common diseases, there is now increasing interest in the common disease-rare variant hypothesis.

The site of injury in VVFD is thought to be the peripheral retina. But recent studies have shown that both central and peripheral retinal areas may be involved (Krauss, et al., 1998; Nousiainen, et al., 2000a; Nousiainen, et al., 2000b; Manuchehri, et al., 2000). However, the question remains as to what is causing injury to the retina. The exact cause or the mechanism is still not known. GABA-T is involved in the catabolism of GABA. If GABA-T activity is reduced, this could lead to an increase in GABA in the retina. When VGB is administered, the level of GABA would rise even more. This could reduce ocular perfusion and glucose metabolism, possibly through ischemia of the retina (Hosking and Hilton, 2002) . If this hypothesis is true, our patients with lower GABA-T activity may have a greater propensity to develop VVFD than their counterparts with higher GABA-T activity. I did not assess the effect of VGB on the GABA-T activity of these patients. In any follow-on study the activity of VGB would have to be assessed along with the GABA-T activity assay to explore the effect of VGB on GABA-T activity in patients who developed VVFD and those who did not. However, Hisama et al. (2001) have shown that there was no change in the GABA levels in patients treated with VGB with VVFD when compared with patients on VGB with normal visual fields, though only three patients with VVFD were studied. However, since GABA is retino-toxic (Izumi, et al., 2004), there could be a threshold above which it induces retinal injury. Izumi et al. (2004) showed that acute exposure of the retina to VGB in presence of light causes retinal injury in rats, while VGB alone did not cause retinal injury. The retina was intact when exposed to GABA in presence or absence of light. However, all of these were acute studies. By contrast, Jammoul et al. (2009) demonstrated that

chronic VGB treatment of rats and mice maintained in 12/12hr night and dark cycles impaired retinal function and caused retinal injury, while rats kept in darkness had similar findings as the control group not treated with VGB.

They also observed that VGB toxicity could be mediated by taurine deficiency. They demonstrated taurine deficiency (67%) in the animals, which had VGB-induced retinal toxicity. Taurine replacement in food reduced the features of retinal VGB toxicity in both rats and mice. The clinical relevance of these findings was evaluated in infants with infantile spasms on VGB treatment. All these patients had significantly lower levels of taurine when compared to their age matched controls. One of the patients had normal taurine concentration before starting VGB. However, after 15 months of treatment with VGB, taurine was undetectable in plasma (Jammoul, et al., 2009). The mechanism of VGB reducing taurine is not clear. GABA could be the culprit again, being a natural substrate of taurine transporters; it could compete with taurine for transport (Debler and Lajtha, 1987). VGB could have a direct action on taurine uptake and release - an explanation for the photo-toxicity to photoreceptors, while GABA was phototoxic only in the presence of light (Jammoul, et al., 2009). Future studies examining the cellular and molecular mechanisms of VGB induced taurine deficiency and photoreceptor degeneration is required for a better understanding of the aetiopathogenesis of VVFD. From the above discussion, it is clear that many factors could be involved in the development of VVFD.

In summary, I have identified lowered GABA-T activity in the platelets of patients with VVFD when compared to patients without VVFD. A limitation of our study is the small numbers of patients studied, and this finding therefore needs replication in

a large set of patients, preferably in a prospective study. An alternative way of validating this phenotypic finding may be through the demonstration of rare variants in the ABAT gene – this work is currently on going.

Chapter 4

Development and validation of an assay to simultaneously determine clobazam and its active metabolite N-desmethylclobazam by HPLC

4.1. Introduction

CLB is a 1, 5-benzodiazepine synthesised in 1972 (Weber, et al., 1972). It has been used successfully to treat many types of epilepsies especially carbamazepine (CBZ) resistant epilepsy. NDCB, the most important and active metabolite of CLB is formed by the action of CYP2C19 on CLB. Drug inducers can increase the activity and drug inhibitors inhibit the activity of CYP2C19 (Dalby, 2004). During long term treatment, NDCB accumulates in the body reaching almost 10 times the concentration of the parent drug (Caccia, et al., 1980; Haigh, et al., 1987) . This may contribute to the efficacy and toxicity of CLB. The concentration of CLB is proportional to the dose in an individual patient but exhibits great inter-individual variation. However the concentration of NDCB is not proportional to the CLB dose but also exhibits great inter-individual variation (Guberman, et al., 1990; Bardy, et al., 1991). Therefore simultaneous estimation of CLB and its metabolite NDCB may be useful for several reasons: (a) to assess compliance; (b) to determine the ratio between the parent drug and the active metabolite as a marker of CYP2C19 activity; (c) to assess inter-individual variability in CLB: NDCB ratio; (d) to relate this variability to genotype; and (e) to assess the possible relationship between CLB/NDCB levels and adverse drug effects.

There are various techniques described for the quantification of CLB and NDCB. These include radiochemical techniques, fluorometry (Stewart, et al., 1979), mass spectrometry, gas chromatography (Badcock and Zoanetti, 1987) and HPLC (Scholten, et al., 1980; Brachet-Liermain, et al., 1982; Ratnaraj, et al., 1984; Dusci and Hackett, 1987; Gazdzik, et al., 1989). In radiochemical techniques, the measured radioactivity represents the total amount of drug, including its metabolites. In fluorometry, the metabolites also contribute to the signal generated to measure the

quantity of the drug. Mass spectrometry, gas chromatography and HPLC are more specific methods (Rupp, et al., 1979). HPLC is less laborious, simple and more cost effective than gas chromatography. For instance, the analyte requires derivatization and has to be vaporized in gas chromatography (Proença, et al., 2004).

The main advantages of HPLC over gas chromatography are lower temperatures during analysis that reduces the probability of isomerization of double bonds, and the possibility of collecting fractions for further analysis (Czauderna and Kowalczyk, 2001). HPLC and liquid chromatography-mass spectrometry (LC-MS) have largely replaced gas chromatography from the bio/drug analytical scene. Mass spectrometry is more sensitive and selective than HPLC, but expensive. There are a number of HPLC methods published for the simultaneous estimation of CLB and NDCB (Brachet-Liermain, et al., 1982; Ratnaraj, et al., 1984; Zilli and Nisi, 1986; Dusci and Hackett, 1987; Gazdzik, et al., 1989; Streete, et al., 1991). Some need a large volume of plasma (1ml) for the estimation of the analytes (Brachet-Liermain, et al., 1982; Ratnaraj, et al., 1984; Dusci and Hackett, 1987). Other HPLC methods are cumbersome to perform (Scholten, et al., 1980; Ratnaraj, et al., 1984) and some use diethyl ether which is inflammable (Brachet-Liermain, et al., 1982; Tomasini, et al., 1985; Dusci and Hackett, 1987). I tried replicating the method published by Streete et al. (1991). I could not procure the internal standard (IS) they used, in spite of contacting the manufacturer. Though other chemicals were used as internal standards, adequate resolution of the peaks of the analytes and ISwas not achieved. Therefore, I chose to develop an alternative simple, sensitive, reproducible and cost effective reverse phase gradient HPLC method to analyse CLB and NDCB simultaneously in patient samples.

The aim of this chapter was to:

To develop and validate a novel, sensitive, reproducible, rapid and simple reverse phase HPLC method for the simultaneous estimation of CLB and NDCB.

4.2. Methods

4.2.1. Reagents and chemicals

NDCB was a kind gift from Centaur Chemicals Pvt. Ltd, Mumbai, India. HPLC grade acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Loughborough, UK) and ammonium acetate from VWR Laboratory Supplies (Poole, UK). CLB, diazepam, Tris-base, chloroquine, primaquine, clozapine and dichloroethane were purchased from Sigma-Aldrich (Poole, UK).

Blank plasma was purchased from the blood bank. This was stored at -20°C. Water from a water purification system (Purelab Option, Elga labWater, High Wycombe, UK) was used throughout the experiments.

4.2.2. Equipment

The HPLC system used consisted of a Dionex P680 pump, a Dionex ASI-100 automated sample injector, a Dionex UVD170U variable wavelength detector set at 235nm wavelength and a computer with Dionex Chromeleon® (version 6.70) data acquisition software.

Separation of CLB and NDCB was initially attempted using a thermo 5µm, 150×4.6 mm Hypersil® BDS C18 column. However, the results of this method were not satisfactory, and therefore a gradient method was developed for the separation of

CLB and NDCB. A Waters, Nova-pak®, C18 (4µ, 3.9×150 mm) column was used in this method.

4.2.3. Sample preparation

Prior to extraction, the calibrators, quality controls (QCs) and samples were taken out from the freezer and allowed to thaw completely. They were vortexed (2 seconds (sec) and centrifuged (1000 rpm for 1min). All calibrators, QCs and samples were run in duplicate. The standard curves for CLB (25, 50, 100, 200, 500ng/ml) and NDCB (200, 400, 800, 1600, 4000 and 8000ng/ml) were prepared by diluting 1000ng/ml CLB and 8000ng/ml NDCB, with drug free plasma in a 10ml labeled glass tube. 500µl of each test sample and QC samples were transferred into a 10ml labeled LSL glass tube. 250µl of Tris buffer (0.8M, pH 10.9) and 25µl of the ISat 20µg/ml were added to each test tube, followed by 6ml of 1, 2-dichloroethane. Diazepam was the ISused for the gradient method. Clozapine, chloroquine and primaquine were experimented as internal standards by the isocratic method. Each tube was then whirl mixed for a minute, followed by rota mixing for 10 min. These tubes were centrifuged (4000 rpm) for 10 min. The supernatant was then discarded carefully by a Pasteur pipette. Great care was taken to avoid shaking and therefore contamination with the lower clear layer. This lower clear layer was transferred into another clean identically labeled 10ml LSL tube, which was dried under a stream of nitrogen gas in a water bath at 30°C. The residue was re-suspended in 100µl of ammonium acetate 50mM (pH 5), which is a part of mobile phase, vortexed (2 sec), centrifuged (1000 rpm for 1min) and transferred to an auto sampler vial. 30µl of the sample was injected into the column. A summary of this procedure is shown in Figure 4.1.

Extraction Procedure

500 µL (Standard, QC or sample)

↓

Tris buffer – 250 µL (0.8M, pH 10.9)

+

Diazepam (IS) –25µl at 20µg/ml in methanol

+

1,2 Dichloroethane – 6mL

↓

Whirl mixed- 20 sec

rota mixing- 10 min

Centrifuge- 4000 rpm for 10 min

↓

Transfer the top layer of cloudy liquid into a tube (This is discarded)

Transfer 5 ml of clear organic phase (the layer below) into a clean tube.

Evaporate till dry under constant flow of nitrogen, whilst held in a water bath at 30°C.

↓

Once dry – reconstitute the residue in the tube with 100 µl of ammonium acetate 50mM (pH 5), which is part of the mobile phase

Vortex – 20 sec

Centrifuge-2000 X *g* for 2 min.

↓

Transfer 100 µl in to HPLC vial.

Inject 30 µl on to the column.

Figure 4.1. Summary of the extraction procedure used to isolate clobazam and N-desmethylclobazam from plasma

4.2.4. Preparation of stock solutions

Stock solutions of CLB, NDCB, diazepam, clozapine, primaquine, and chloroquine (1mg/ml each) were prepared by dissolving the appropriate amount of the compound, in HPLC grade methanol. These stock solutions were stored at 4°C. Every 6 months fresh stock solutions were prepared for use.

4.2.5. Preparation of calibrators, quality controls and internal standard

The stock solutions of CLB and NDCB were diluted by drug free plasma to obtain a concentration of 1000ng/ml and 8000ng/ml respectively. On the day of the experimental setup, the different points (calibrators) on the standard curve for CLB (25, 50, 100, 200, 500ng/ml) and NDCB (200, 400, 800, 1600, 4000 and 8000ng/ml) were prepared by diluting 1000ng/ml (CLB) and 8000ng/ml (NDCB) with drug free plasma.

The QCs were constituted by diluting the stock solutions (1mg/ml) of CLB and NDCB with drug free plasma. Three QCs of CLB were 70, 300 and 762.15ng/ml and that of NDCB were 600, 2000 and 6097.56 ng/ml. They were called low quality control (LQC), medium quality control (MQC) and high quality control (HQC), respectively. They were selected as follows: The LQC was 5-10% of the maximum concentration of CLB and NDCB on the standard curve; the MQC was 25-30% of the maximum concentration of CLB and NDCB on the standard curve; and HQC was 75-80% of the maximum concentration of CLB and NDCB on the standard curve. All these were stored in aliquots of 1.2ml at -20°C. For daily use, 20µg/ml of diazepam (IS) was prepared from a 1mg/ml stock solution, and was used as the internal standard.

4.2.6. Chromatographic conditions

Initially, a modification of the method described by Streete *et al* (1991) was used. This was a reverse phase isocratic HPLC method. The mobile phase at pH 5.5 consisted of 1 litre acetate buffer (made by adding 50 ml of 1M sodium hydroxide (NaOH) and 58 ml of 1M acetic acid and making up the volume to 1L with distilled water) and 580 ml of ACN. The flow rate was 1 ml/min. To summarise, the mobile phase concentration and pH were altered in an attempt to obtain peaks with good resolution and properties for CLB, NDCB and the IS. Clozapine, primaquine and chloroquine were tried as IS. The molecular weight and structures (Figure 4.2) of these compounds were similar to that of CLB and NDCB. The pH of the mobile phase was altered from 8-3.6, by adding glacial acetic acid. The concentration of ACN in the mobile phase was changed from 5%- 40%.

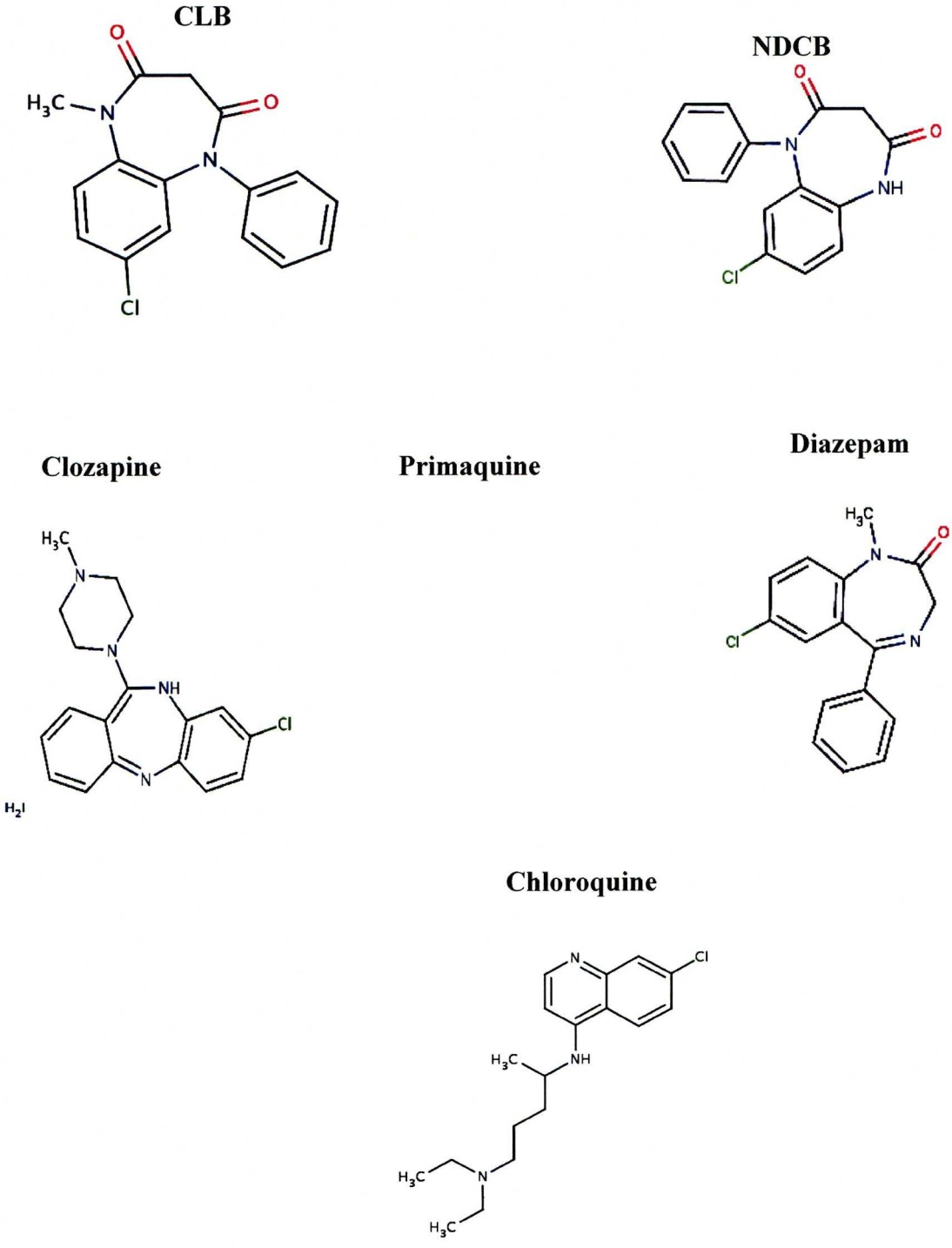


Figure 4.2. Chemical structures of clobazam (CLB), N-desmethylclobazam (NDCB) and the drugs tried as internal standards

Since the above method did not produce peaks (of the IS, CLB and NDCB) with good resolution, a gradient method was developed for the quantification of CLB and NDCB. Various mobile phase gradients (Tables 4.1 A-C) were used to obtain peaks (CLB, NDCB, IS) of good resolution. Primaquine, clozapine and diazepam were used as IS. Finally, diazepam was selected as the IS for this method. CLB, NDCB and diazepam (IS) were eluted from the Waters column by a gradient mobile phase (ammonium acetate 50mM, ACN + 0.05%formic acid, pH 5) at a flow rate of 1ml/min. The gradient program used is shown in Table 4.1C. The eluant from the column was monitored at 235nm. The column was maintained at a temperature of 21°C during the run. A summary of the optimal conditions for the analysis is given in Table 4.1C and Table 4.2.

Table 4.1. An outline of the mobile phase gradient programs consisting of ammonium acetate 50mM (pH=5) and acetonitrile (ACN) buffer to elute clobazam and N-desmethylclobazam. Program C gave peaks with good resolution and properties and was used to estimate the levels of CLB and NDCB in all patient samples.

A

Time (min)	**Ammonium acetate 50mM (pH=5) (%)**	**ACN + 0.05%Formic acid (%)**
0	10	90
10	100	0
15	10	90
20	10	90

B

Time (min)	Ammonium acetate 50mM (pH=5) (%)	ACN + 0.05%Formic acid (%)
0	20	80
10	100	0
15	20	80
20	20	80

C

Time (min)	Ammonium acetate 50mM (pH=5) (%)	ACN + 0.05%Formic acid (%)
0	30	70
10	100	0
15	30	70
20	30	70

Table 4.2. HPLC characteristics for the gradient run of reverse phase HPLC

Column	Mobile Phase preparation	Chromatograph	Detector wavelength	Auto sampler	Integration
Guard column: Thermo®, Column Saver Column: Waters, Nova-pak®, C18 4µm, 3.9×150 mm Temperature: 21°C	50mM pH 5 gradient (Table 4.1 C)	Flow rate: 1 ml/min Run Time: 20 minutes	235 nm	Injection Volume: 30 µl	Data handling: Dionex Chromeleon™ Peak measurement: Area

4.2.7. Method validation

This assay was validated for accuracy and precision for both CLB and NDCB. Accuracy is a measure of how close one can get to the true value with the assay method. It was evaluated by calculating percentage bias [% bias; [(measured concentration-nominal concentration)/ nominal concentration] × 100]. The precision is the measure of how close the data values are to each other from a number of measurements under the same analytical conditions and is generally expressed in terms of the percentage coefficient of variation (CV%) [(Std. Deviation /mean) × 100]. Inter-assay and intra-assay accuracy and precision were evaluated from low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples. To determine intra-assay accuracy and precision, 6 replicate sample analyses were performed on one run. Inter-assay accuracy and precision was determined over a period of 6 separate days. 6 runs of standard curves and QCs were done on these days. The percentage bias and %CV were estimated for these runs. Accuracy and precision, determined at each concentration level, should not exceed 15%, except for LQC, where it should not exceed 20% (FDA, 2001). The linearity of the method was evaluated over the concentration range of 25-1000ng/ml for CLB and 200-8000ng/ml for NDCB.

The upper limit of quantification was set as the top point on the standard curve. The lower limit of quantification was defined as the lowest concentration that produced a peak with peak to noise ratio more than 3, where the peak area ratio had a CV% less than 20%. The lower limit of detection was taken as the lowest concentration which produced a peak whose height is three times the background noise (FDA, 2001).

4.2.8. Stability and recovery

Previous studies have reported the stability of CLB and NDCB under various conditions (Streete, et al., 1991; Rouini, et al., 2005). Patient samples received in our lab underwent centrifugation at 2600 X *g* and the resulting plasma was stored as aliquots of 1.2-1.8mls, at -20°C until analysed. These samples were completely thawed before being processed for HPLC analysis (as mentioned above for the QCs and calibrators). It is important to check whether the samples are stable under these conditions. So the QCs- LQC, MQC and HQC were analyzed in duplicates on 3 separate occasions to determine the plasma concentrations of CLB and NDCB under the 4 different storage conditions (stored at -20°C, left at room temperature for 48 h prior to analysis, subjecting samples to three freeze-thaw cycles and storage for 7 days in the refrigerator at 4 °C prior to analysis).

Recovery (extraction efficiency) of CLB and NDCB after the liquid–liquid extraction was determined by comparing peak areas of each compound in extracted plasma to those of non-processed standard solutions. QCs - LQC, MQC and HQC of CLB and NDCB were used for this. Six replicates of each of the QCs on 3 different occasions were done. For the liquid–liquid extraction, drug free plasma was spiked with CLB and NDCB to obtain the QCs, as mentioned above. 500µl of the calibrator/QC/patient plasma is taken and processed and reconstituted in 100 µl of mobile phase. For direct injections, 500 µl of each of the QCs in methanol was dried with liquid nitrogen and reconstituted in 100 µl of mobile phase. Percentage recovery was calculated by evaluating the resulting peaks.

4.2.9. Data analyses

The peaks were integrated using Dionex Chromeleon™. The peak areas of the standards or QCs or samples, and that of the IS were used for further calculations. A calibration/standard curve was constructed using peak area ratios (PAR) (peak area of CLB or NDCB/peak area of IS) of the calibrators by applying linear regression analysis using Microsoft Excel, 2003 (Microsoft, USA). All concentrations of unknowns and QCs were then calculated from their PARs against the calibration line.

One-way analysis of variance (ANOVA) with Bonferroni correction was used to assess the stability of CLB and NDCB under the following conditions: stored at -20°C, left at room temperature for 48 h prior to analysis, subjecting samples to three freeze-thaw cycles and storage for 7 days in the refrigerator at 4°C prior to analysis. A p value < 0.05 indicated a significant effect of the treatment/storage condition on the stability of CLB and NDCB.

Percentage recovery of CLB and NDCB was calculated as follows: (peak area of CLB or NDCB extracted from plasma / peak area of CLB or NDCB from direct injection) x 100.

4.2.10. Measurement of clobazam and N-desmethylclobazam in patient samples

The gradient method outlined above was used to determine the concentration of CLB and NDCB in epilepsy patients. These patients were recruited from different centers in UK (see chapter 5 for details of ethics and consent procedures). Blood samples were collected in a 9 ml (EDTA) tube. Plasma was separated by centrifugation at

2600 X *g* for 20-30 minutes. Aliquots (1.2 -1.8ml) were stored at -20°C until analyzed.

4.3. Results

4.3.1. Detection & chromatography by the isocratic reverse phase HPLC method

CLB, NDCB, clozapine, chloroquine, and primaquine were detected by UV-HPLC at 235nm.

4.3.1.1. Detection by the isocratic method

In spite of changing the assay conditions - pH of the buffer, constitution of the buffer and the column, I was unable to attain good separation between CLB, NDCB and clozapine, chloroquine, and primaquine (the internal standards) used. The results can be summarised as follows:

(1) The peak of chloroquine was close to the injection peak when the conditions were adjusted to get the peaks of CLB and NDCB (Figure 4.3 A). If the concentration of ACN was increased to shift the peak of chloroquine to the right, then the retention times of CLB and NDCB were more than 20 minutes (Figure 4.3 B). If the retention times of the drugs are more than 20 min, the run time will be longer, consuming more mobile phase and the number of patient samples, which could be analysed, will be less.

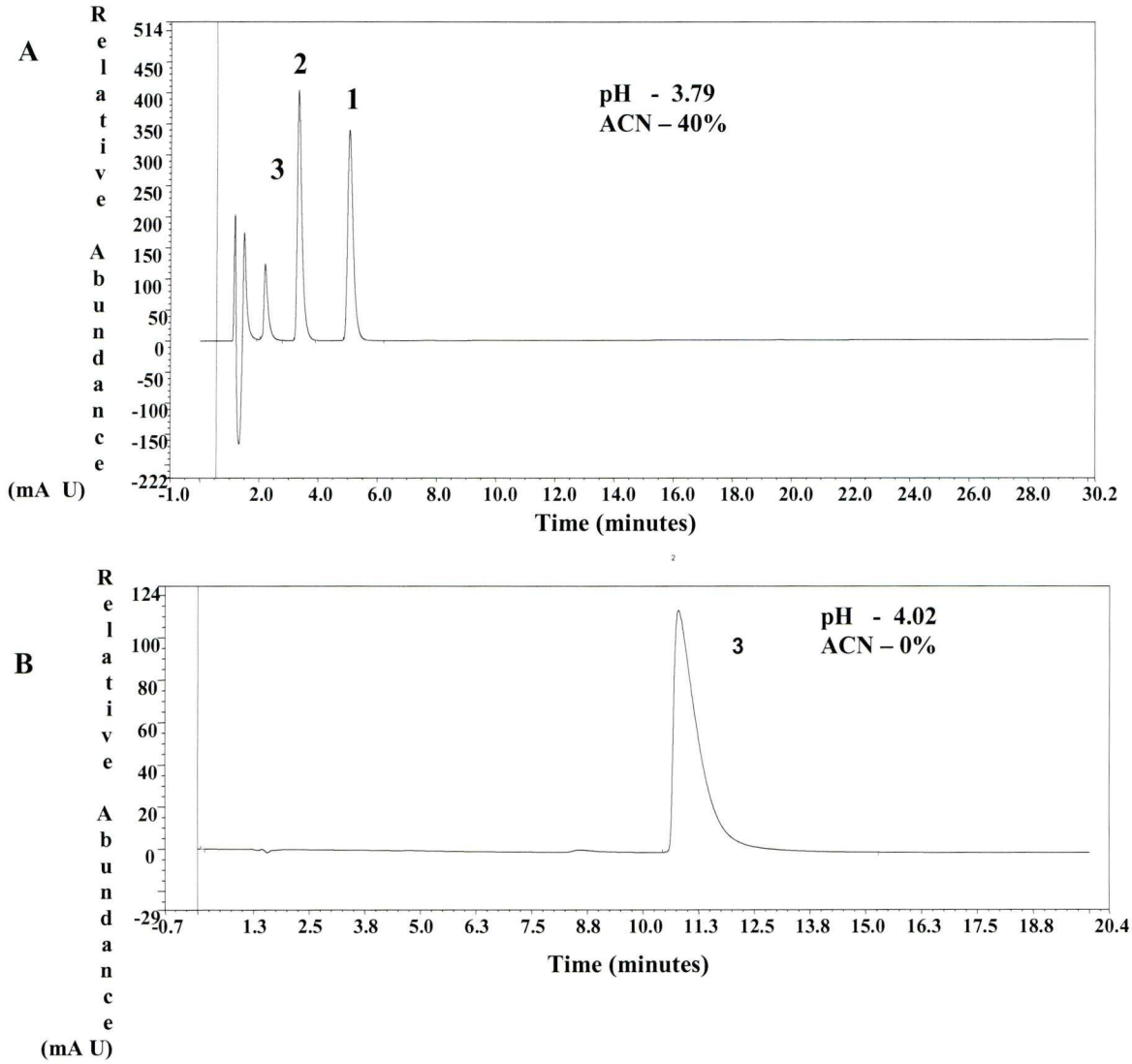


Figure 4.3. Chromatogram showing chloroquine used as internal standard under the isocratic HPLC conditions. 1 = Clobazam, 2 = N-desmethylclobazam, 3 = Chloroquine.

In the case of clozapine and primaquine, though resolution was obtained under some of the conditions tried (Figure 4.4 A & 4.5 A), when CLB, NDCB and these drugs were extracted from plasma, the resolution of the peaks disappeared (Figure 4.4 B & 4.5 B). With primaquine, there was a significant peak fronting, which was due to impurities in the compound (4.5 C). These impurities are produced during the manufacture of primaquine and are present in the product I purchased (Dongre, et al., 2008).

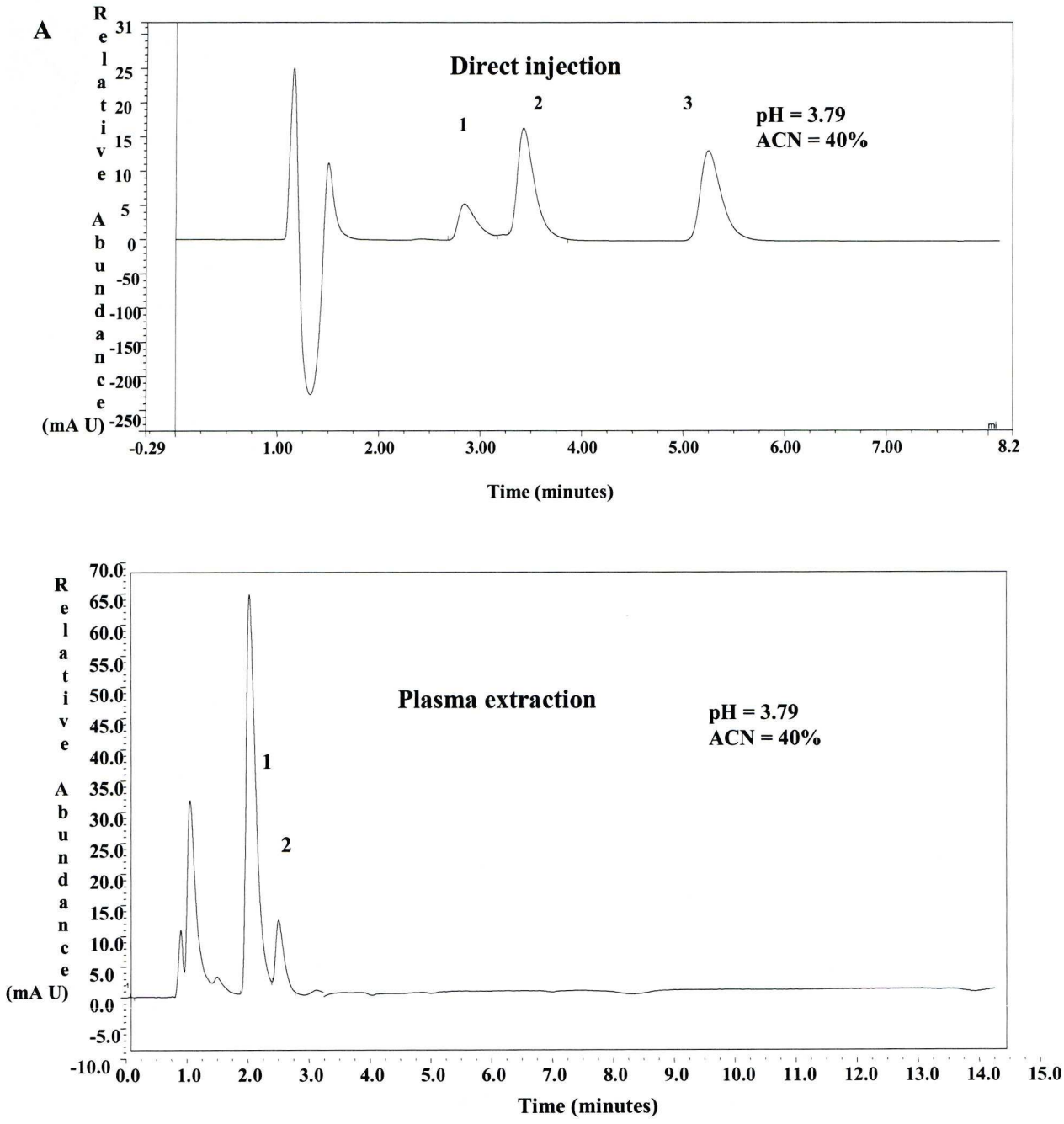


Figure 4.4. Chromatogram showing clozapine used as internal standard under the isocratic HPLC conditions. 1= clozapine; 2 = N-desmethylclobazam; 3 = clobazam.

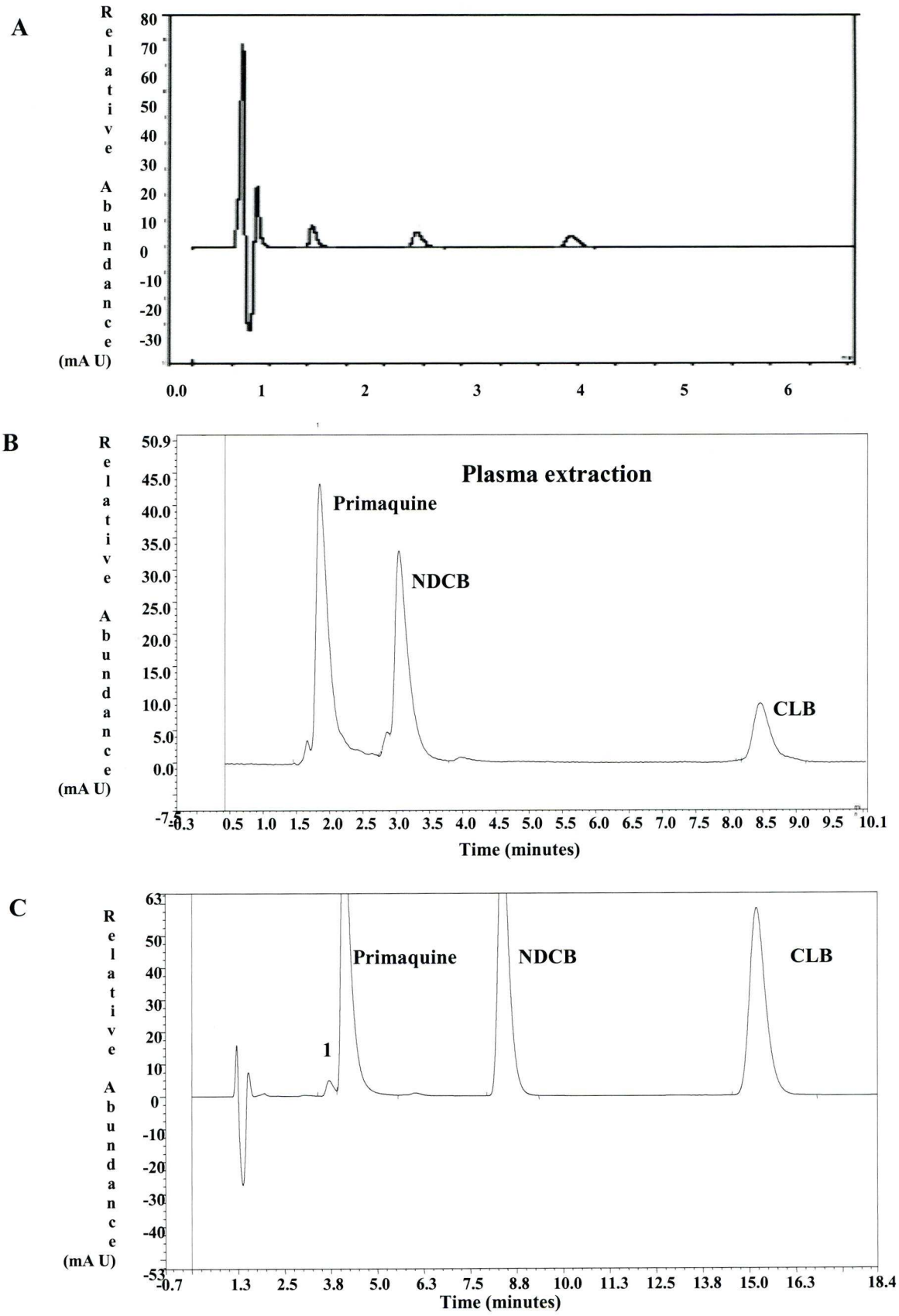


Figure 4.5. Chromatograms showing primaquine under the isocratic HPLC conditions. 1= front peaking of primaquine.NDCB = N-desmethylclobazam, CLB= clobazam

4.3.2. Detection & chromatography by the gradient reverse phase HPLC method

CLB, NDCB, diazepam, clozapine and primaquine (Figure 4.2) were detected by UV-HPLC at 235nm over a total run time of 20min. With the program shown in Table 4.1 A, CLB and NDCB did not elute in 20 minutes (Figure 4.6 A & 4.6 B), while with the program shown in Table 4.1B, CLB did not elute in 20 min (Figure 4.6C). The peak of clozapine was too close to that of NDCB and failed to give good resolution (Figure 4.7 A). With the gradient program shown in Table 4.1C, the impurity in primaquine was separated; however, the blank plasma had peaks with similar retention times as primaquine (Figure 4.7 C). Drug free plasma did not interfere with the peaks of CLB, NDCB, clozapine (Figure 4.7 A) and diazepam (Figure 4.8 A-C). The typical retention times were 8.29, 7.5, 10.71, 10.30, 7.10 and 6.25 min for CLB, NDCB, diazepam, clozapine and primaquine respectively, using the gradient program shown in Table 4.1C.

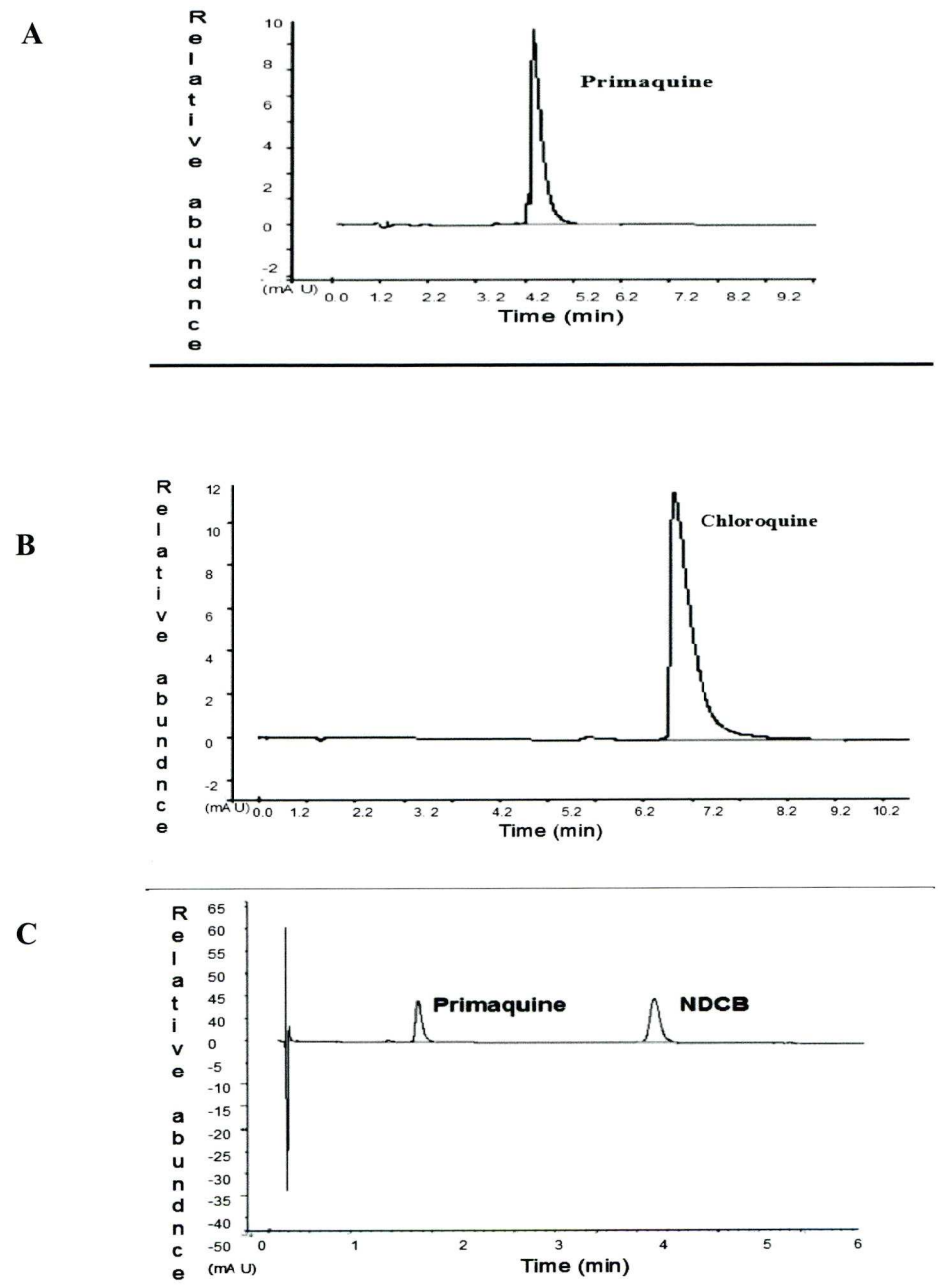


Figure 4.6. Chromatograms of clobazam (CLB) and N-desmethylclobazam (NDCB) using primaquine (A) and chloroquine (B) as internal satandard. CLB and NDCB failed to elute with the gradient program-Table 4.1A, while CLB did not elute with gradient program – Table 4.1B.

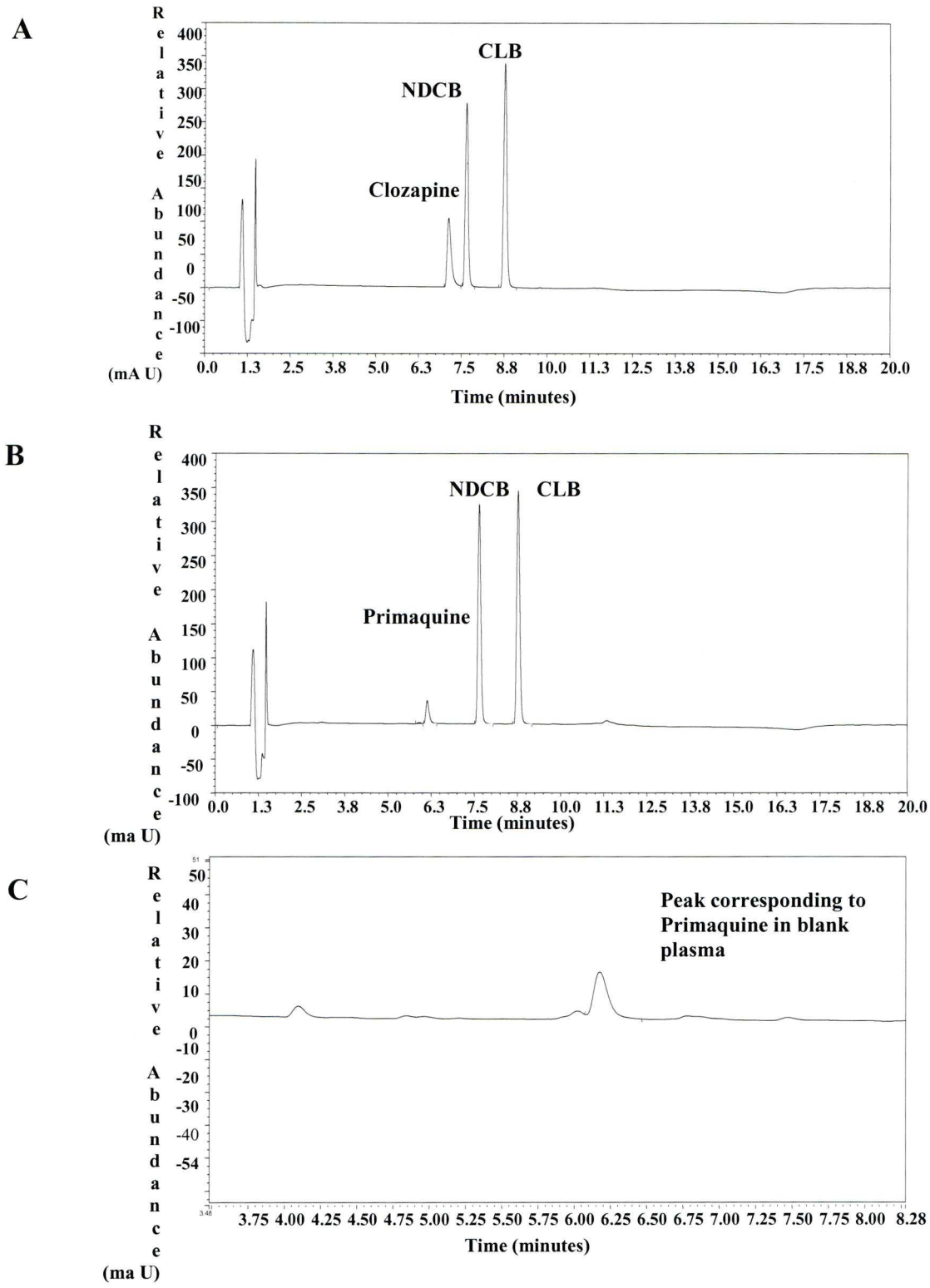


Figure 4.7. Chromatograms of clobazam (CLB) and N-desmethylclobazam (NDCB) using clozapine (A) and primaquine (B) as internal standard. The impurity in primaquine is separated (B). C demonstrates the peak in blank plasma interfering with the peak of primaquine.

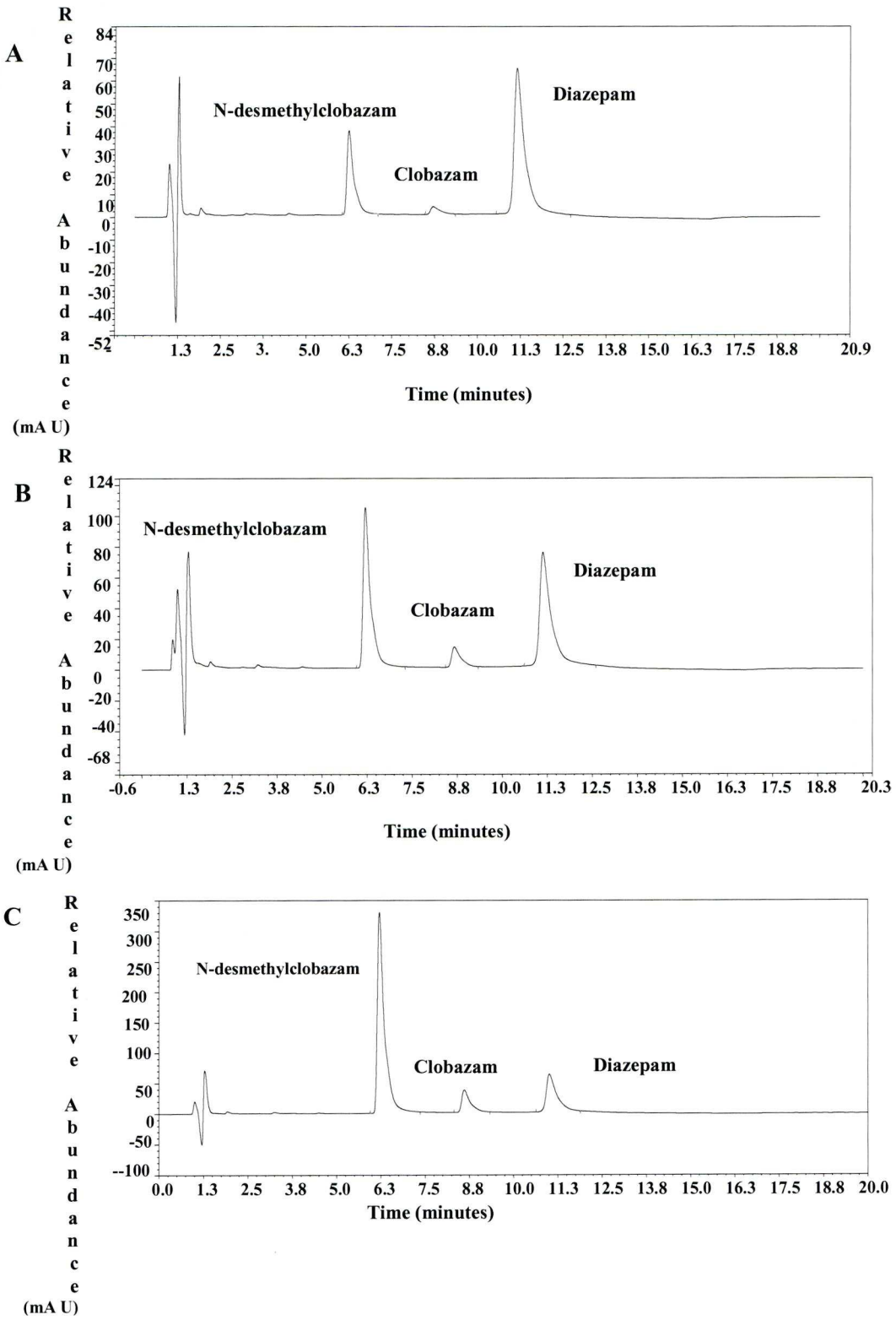


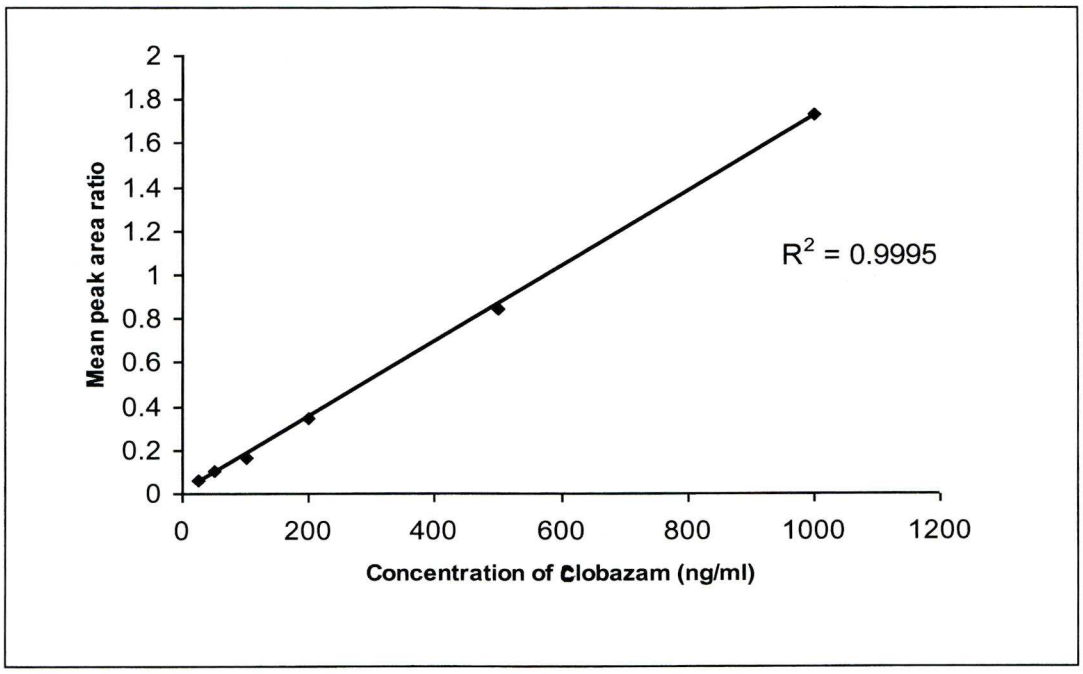
Figure 4.8. Representative chromatograms of clobazam and N-mesmethylclobazam Low quality control (LQC) (A), medium quality control (MQC) (B) and high quality control (HQC) (C) with diazepam used as internal standard

4.3.3. Assay validation

Six standard curves and QC samples (in duplicate) were performed for the validation of this assay. The standard curves used for the quantification of CLB and NDCB were linear over the concentration range of 100-1000ng/ml and 200-8000ng/ml, respectively. They were adequately described by a non-weighted linear regression. Correlation coefficient (r^2) for all validation standard curves was above 0.999. Examples of standard curves used to determine CLB and NDCB concentrations are shown in Figure 4.9.

The upper limit of quantification was set as the top point on the standard curve. It was 1000ng/ml for CLB and 8000ng/ml for NDCB. The lower limit of quantification of CLB was 12.5ng/ml and that of NDCB was 25ng/ml. The lower limit of detection of CLB was 5ng/ml and that of NDCB was 15ng/ml.

A



B

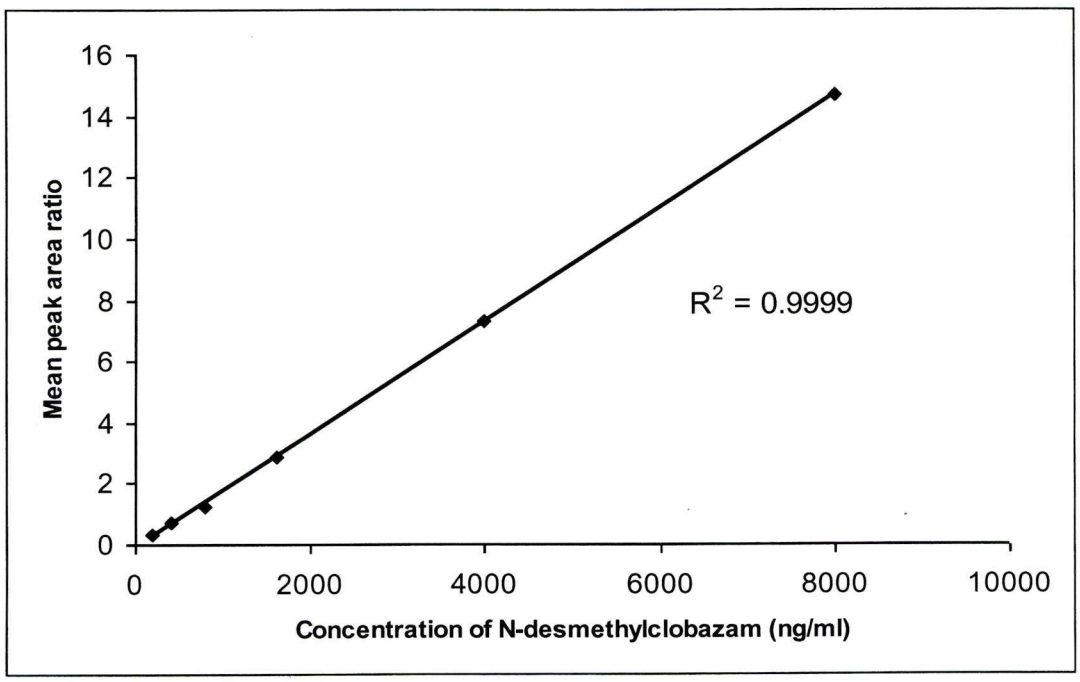


Figure 4.9. Plots of standard curves to determine the concentration of (A) clobazam and (B) N-desmethylclobazam

Intra and inter-assay accuracy and precision of CLB and NDCB were evaluated by assaying quality control samples at three concentration levels (LQC, MQC, and

HQC) (Tables 4.3 and 4.4). Intra and inter-day coefficients of variation were less than 7% for both CLB and NDCB. The intra and inter assay accuracy (% bias) for all QC concentrations was less than 6% for both CLB and NDCB.

Table 4.3. Intra-assay precision and accuracy of the low (LQC), medium (MQC) and high (HQC) quality control samples of clobazam and N-desmethylclobazam as measured by the HPLC method (n=6).

	QC Level	Nominal conc. (ng/ml)	Measured conc. (ng/ml) Mean (SD)	Precision (CV %)	Accuracy % bias
Clobazam	LQC	70	71.84 (3.33)	4.632	2.767
	MQC	300	290.76 (10.39)	3.572	-2.612
	HQC	762.19	790.15 (45.64)	5.776	-4.384
N-desmethyl clobazam	LQC	700	719.37(17.62)	2.448	0.043
	MQC	2000	1947.77(69.64)	3.58	2.612
	HQC	6097.56	5830.23(305.01)	5.231	5.932

Table 4.4. Inter-assay precision and accuracy of the low (LQC), medium (MQC) and high (HQC) quality control samples for clobazam and N-desmethylclobazam as measured by the HPLC method (n=6)

	QC Level	Nominal conc. (ng/ml)	Measured conc. (ng/ml) Mean (SD)	Precision (CV%)	Accuracy % bias
Clobazam	LQC	70	62.759 (4.30)	6.855	-4.599
	MQC	300	296.976 (17.63)	5.935	-1.008
	HQC	762.19	783.025(42.10)	5.377	2.734
N-desmethyl clobazam	LQC	700	677.059 (39.53)	6.850	-3.824
	MQC	2000	1866.259 (105.21)	5.637	-6.687
	HQC	6097.56	5731.981(330.72)	5.770	-5.995

Simultaneous quantification of CLB and NDCB was performed in patient samples using this method. Chromatograms of samples from two patients are shown in Figure 4.10.

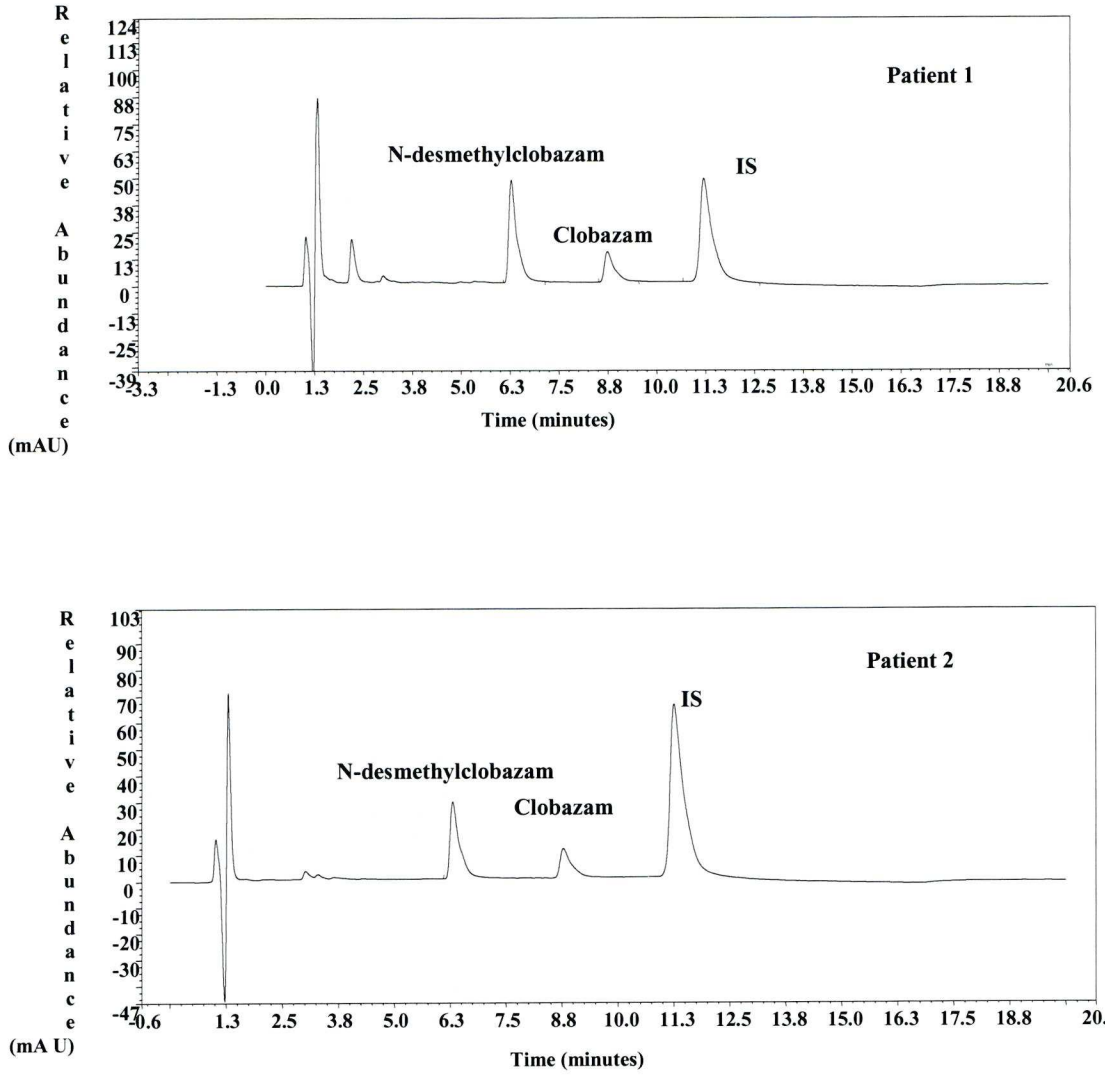


Figure 4.10. Chromatograms representing samples taken from two patients who were on clobazam. IS is the internal standard used (diazepam).

4.3.4. Stability and recovery

Concentrations of QCs obtained at various storage conditions are shown in Table 4.5 and Table 4.6. ANOVA was used to compare the QCs after storing at -20°C, leaving the samples at room temperature for 48 h prior to analysis, subjecting samples to three freeze-thaw cycles and storage for 7 days in the refrigerator at 4°C prior to analysis. There was no significant difference between QCs at a p value ≤ 0.05. That is subjecting the QCs to these conditions did not affect the concentration of CLB or NDCB.

Table 4.5. Comparison of the QCs (low quality control (LQC), medium quality control (MQC) & high quality control (HQC)) of clobazam when analysed in duplicate on 4 separate occasions. Data are expressed as mean concentration in ng/ml ± standard deviation and analysis was performed with one way analysis of variance with Bonferroni correction ($p \leq 0.05$). There was no significant difference between the QC's across the different storage conditions within each category (LQC, MQC & HQC).

Storage conditions	LQC(70ng/ml)	MQC(300ng/ml)	HQC(762.19ng/ml)
Stored at-20°C	66.244 ± 1.62	293.485 ± 8.00	770.073± 45.93
Room temperature for 48 hrs	76.490± 76.49	301.764± 12.74	756.854± 30.17
Three freeze-thaw cycles	79.084 ± 2.63	313.826± 22.37	778.535± 34.08
7 days at 4 °C	69.504± 10.61	302.103± 14.67	727.987± 62.31

Table 4.6. Comparison of the QCs (low quality control (LQC), medium quality control (MQC) & high quality control (HQC)) of N-desmethylclobazam when analysed in duplicate on 4 separate occasions. Data are expressed as mean concentration in ng/ml ± standard deviation and analysis performed with one way analysis of variance with Bonferroni correction ($p \leq 0.05$). There was no significant difference between the QC's across the different storage conditions within each category (LQC, MQC & HQC).

Storage conditions	LQC(700 ng/ml)	MQC(2000ng/ml)	HQC(6097.56ng/ml)
Stored at-20°C	607.832 ± 21.85	1938.314 ± 122.36	5671.598 ± 405.59
Room temperature for 48 hrs	617.794 ± 32.65	2020.386 ± 19.68	5763.191 ± 317.78
Three freeze-thaw cycles	647.511 ± 29.14	1979.913 ± 46.77	5868.172 ± 106.54
7 days at 4 °C	665.571± 9.43	2010.264 ± 28.30	5924.070 ± 232.85

Recovery of CLB and NDCB was above 90% for the 3 concentrations (LQC, MQC and HQC) checked as demonstrated in Table 4.7.

Table 4.7. Percentage recovery from plasma at 3 concentrations,(low quality control (LQC), medium quality control (MQC) & high quality control (HQC) LQC- 70ng/ml, MQC-300ng/ml and HQC-762.19ng/ml of clobazam, and LQC-700 ng/ml, MQC-2000ng/ml and HQC-6097.56ng/ml of N-desmethylclobazam, in duplicates on 3 different occasions, calculated by comparing peak areas of directly injected drug solution and drug that was extracted from plasma by a liquid-liquid extraction method. Data are expressed as mean concentration in ng/ml ± standard error of mean (SEM).

	Percentage recovery (%)		
Drug	**LQC**	**MQC**	**HQC**
Clobazam	96.49 ±0.04	95.64 ± 0.03	94.67 ± 0.038
N-desmethyl-clobazam	95.34 ±0.034	94.67 ± 0.03	95.92 ± 0.038

4.4. Discussion

I have described a novel HPLC method for the quantification of CLB and NDCB simultaneously, which is sensitive, accurate, precise, reproducible, cost effective and simple. I initially attempted to set up a method in the lab using a modification of the method described by Streete *et al.*(1991). At least two columns and different analytical conditions were utilised using isocratic conditions, but the assay could not give good resolution of the peaks of the analytes and IS.

Since I was unable to procure methoxycarbamazepine which was used as the ISin previous studies (Streete, et al., 1991), other structurally related compounds such as clozapine, chloroquine and primaquine were tried as internal standards. But the

isocratic method failed to separate these drugs from CLB or NDCB, as shown in Figures 4.4 and 4.5. This could be due to the fact that all these drugs have similar structures to CLB and NDCB (Figure 4.2). It is understood that the drugs/chemicals to be used as IS should have comparable physicochemical characteristics (Wieling, 2002). Their molecular weights should also be close to each other. All these compounds also demonstrated significant absorbance at 235 nm which was the wavelength used in this method to detect CLB and NDCB. In view of these initial findings, a decision was made to develop a gradient HPLC method for the estimation of CLB and its active metabolite NDCB in patient plasma.

Formic acid was used as a peak modifier in the gradient reverse phase HPLC method. Very low concentrations of an additive to the mobile phase can improve the partition characteristics of the analytes by coating the stationary phase with the additive or additive plus water. This leads to rapid equilibration of the analytes between the mobile phase and stationary phase leading to peaks with better characteristics (Wieling, 2002). Various gradient protocols were used (Table 4.1). Even with the gradient method used (Table 4.1 C), good resolution of clozapine was not achieved (Figure 4.7 A). The gradient method however did separate the impurity in primaquine (Figure 4.7 B), but four samples of blank plasma obtained from the blood bank showed a peak with similar retention time as primaquine (Figure 4.7 C). For these reasons, primaquine and clozapine were rejected as an IS. Other compounds structurally related to CLB could be investigated as IS. However due to lack of time, this was not possible. Therefore, with due considerations of the problems encountered with the other compounds tested as IS, I decided to use diazepam as the IS. Diazepam gave good peak resolution from CLB and NDCB (Figure 4.8 A-C.), with good peak properties. A disadvantage of using diazepam as an IS is that it is

used in status epilepticus, but not as first line treatment. In addition, it can also be used in chronic epilepsy, although this is uncommon. Diazepam also has anti-anxiety properties, but National Institute for Health and Clinical Excellence (NICE) (http://www.nice.org.uk/nicemedia/pdf/CG022NICEguidelineamended.pdf) does not recommend diazepam as the drug of choice for anxiety. Therefore, I felt that the chance of patients being on this drug was small, and therefore its use as an IS was warranted.

Various HPLC techniques have been described for the quantification of CLB and NDCB as mentioned in the introduction to this chapter. The HPLC method described here uses only 500µl of plasma. The extraction procedure used is simple and based on that reported by Streete *et al.* (1991). It is a single step liquid-liquid extraction. The procedure is simplified by substituting a filtering step mentioned in Streete *et al.* (1991), with centrifugation, which is much easier and faster to perform. This method gave pure chromatograms without any interfering peaks, i.e. it was able to extract the pure drug and its metabolite, as shown in Figures 4.8 A-C. The advantage here is that it enabled us to analyse 40-50 samples per day (24hrs).

Plasma was separated from the blood samples and stored in -20°C until analysed. On the day of analysis, the samples were thawed fully before analysis. This procedure did not affect the stability of CLB and NDCB, with recovery being above 90%, which is an improvement on previous methods (Dusci and Hackett, 1987; Streete, et al., 1991).

A minimum of six assays were prepared and analysed to obtain mean values for calibration (standard curve points) and QC analysis. This compensates for the variation in the preparation of stock solutions, calibrator points and QCs. The assay

performance was monitored by the QCs. The accuracy of this HPLC method in quantifying CLB and NDCB was between 4 and 7% which is within acceptable limits (FDA, 2001). The inter-assay and intra-assay precision did not exceed 7 and 6%, respectively. This is comparable with some studies (Tomasini, et al., 1985; Kunicki, 2001) and better than others (Pistos and Stewart, 2003).

In patients, the concentrations of the drug and metabolites show wide inter-individual variation (Guberman, et al., 1990; Bardy, et al., 1991). Given current clinical practice in the use of CLB, most epilepsy patients will be on other AEDs. These drugs could be both inducers (phenytoin, phenobarbitone, carbamazepine) or inhibitors (valproic acid) of CYP2C19, and thereby alter the levels and the ratio of CLB and NDCB. Genetic factors could also have a similar effect. Therefore, it is vital that the assay can detect and quantify a wide range of concentrations of CLB and NDCB. The range of CLB and NDCB, used in the various HPLC methods, reported in literature varied as well, ranging from <200ng/ml to 896ng/ml for CLB and <500ng/ml to 7,058ng/ml for NDCB. The range of concentrations on the standard curves was carefully selected after considering the reported plasma concentrations of CLB and NDCB in patients on CLB (Brachet-Liermain, et al., 1982; Dusci and Hackett, 1987; Streete, et al., 1991; Knapp, et al., 1999; Kunicki, 2001). Thus the standard curve utilised had 6 points ranging from 25-1000ng/ml for CLB and 100-8000ng/ml for NDCB.

Since CLB is used as an adjunct in the treatment of refractory epilepsy patients (Dalby, 2004), these patients will be on other AEDs. Ideally, I should have explored whether the peaks of other AEDs interfere with that of CLB. However, due to lack of time, I could not do this.

In conclusion, a simple, fast, sensitive, reproducible HPLC method with good accuracy and precision for the quantification of CLB and NDCB was developed and validated. This method was used to estimate the concentrations of CLB and NDCB in patient samples, the full results of which are presented in chapter 5.

Chapter 5

The impact of CYP2C19 polymorphisms on the metabolism of clobazam, its efficacy and toxicity

5.1. Introduction

CLB, a 1,5-benzodiazepine (Figure 5.1 a) is acclaimed for its better potency and side effect profile than its predecessors - the 1,4 benzodiazepines (e.g. diazepam-Figure 5.1 b). The shift of the nitrogen from position 4 to position 5 in the heterocyclic ring (as shown in Figure 5.1) is responsible for its better anticonvulsant properties and less sedative and muscle relaxant properties (Dichter and Brodie, 1996). NDCB, the major metabolite of CLB has a longer half-life than the parent drug. It attains 8-10 times higher steady-state concentrations than CLB, thereby exerting an important influence on efficacy and the development of adverse drug effects (Fielding and Hoffmann, 1979; Greenblatt, et al., 1981).

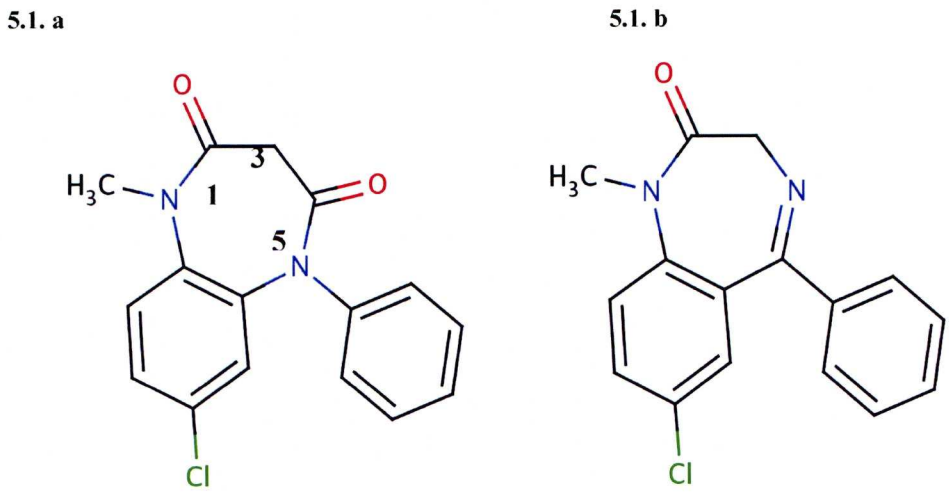


Figure 5.1. Chemical structures of anti-convulsants (a) clobazam and (b), diazepam

Despite its established efficacy, CLB remains under-used because of concerns regarding development of adverse drug effects and tolerance (Shorvon, 1998). Side effects due to CLB have been reported in 20 to 85% of the clinical trials, but only around a third of these were significant leading to change in dose or termination of

treatment (Koeppen, et al., 1987). Drowsiness is the most common adverse effect reported affecting around 26% of patients followed by dizziness (7%), dry mouth (3%), headache (2.1%), constipation (2.1%) and nausea (1.6%) (Koeppen, 1985; Shorvon, 1995).

There is preliminary evidence suggesting that genetic variation in *CYP2C19* affects the metabolism, efficacy and adverse effects of CLB (Seo, et al., 2008). CLB is primarily metabolized to its active metabolite NDCB by CYP3A4 and CYP2C19 in humans. NDCB is later converted to 4-hydroxy nor-clobazam mainly by CYP2C19 (Giraud, et al., 2004). The concentration of CLB and NDCB shows great inter-individual variation (Guberman, et al., 1990; Bardy, et al., 1991). Co-medications that are inducers or inhibitors of CYP3A4 and CYP2C19 are partially responsible for this. Since CLB is converted to NDCB, primarily by CYP3A4 and CYP2C19, inducers or inhibitors could decrease or increase the levels of NDCB respectively (Kosaki, et al., 2004; Seo, et al., 2008). The SNPs in CYP3A4 are unlikely to contribute substantially to inter individual variability in CYP3A4 activity due to their limited functional significance or low allelic frequency (Lepper, et al., 2005). In addition, the serum concentrations of NDCB in patients who are not on treatment with CYP3A4 inducers are not predictable by CLB dose alone.

Many polymorphisms in *CYP2C19* affect the function of CYP2C19. CYP2C19*2 and CYP2C19*3 decrease the activity of CYP2C19 (De Morais, et al., 1994; Poolsup, et al., 2000). Based on *CYP2C19* genotypes, individuals can be divided in 3 sub groups namely: homozygous extensive metabolizers (EMs –CYP2C19 *1/*1), heterozygous extensive metabolizers (CYP2C19 *1/*2 or CYP2C19 *1/*3, CYP2C19 *2/*3) and poor metabolizers (PMs- CYP2C19*2/*2, CYP2C19 *3/*3,

CYP2C19 *2/*3) (Gardiner and Begg, 2006). While the majority of SNPs inhibit the activity of CYP2C19, CYP2C19*17 increases CYP2C19 activity (Sim, et al., 2006). The frequency of PMs varies in different populations. It ranges from 13 to 23% in Asians and 1-8% in Caucasians (Gardiner and Begg, 2006). CYP2C19*2 and CYP2C19*3 account for 85% of Caucasian PMs and 99% of Asian PMs (De Morais, et al., 1994); i.e, in the remaining patients, other mutations may be important.

CYP2C19*2 (rs4244285) allele is a single base pair mutation (guanine to adenine) in exon 5 of *CYP2C19* (De Morais, et al., 1994). This results in an aberrant splice site that changes the reading frame of the mRNA and a premature stop codon 20 base pairs downstream. Another SNP (rs4986893) (G to A) in position 636 of exon 4 of *CYP2C19* (CYP2C19 *3) also produces a premature stop codon. Thus both of these SNPs create a truncated non-functional protein (De Morais, et al., 1994). The allele CYP2C19*17 contains 2 SNPs -806C>T (rs12248560) and -3402C>T in the 5' flanking region of *CYP2C19* gene. CYP2C19*17 binds nuclear proteins and facilitates increased gene expression in mice (Sim, et al., 2006). Hence CYP2C19*17 could increase the metabolism of drugs metabolized by CYP2C19.

It is known that *CYP2C19* genotype is a major determinant of NDCB concentration (Kosaki, et al., 2004; Giraud, et al., 2004; Seo, et al., 2008). There are very few studies, which explored the impact of *CYP2C19* on the metabolism, efficacy and development of adverse drug effects to CLB. Two of them were conducted in Japanese patients (Kosaki, et al., 2004; Seo, et al., 2008). Only one study looked at the effect of *CYP2C19* genotype on metabolism of CLB in Caucasian population (Giraud, et al., 2004). Since the polymorphisms vary among populations, it is

essential to explore the effect of *CYP2C19* genotype on NDCB concentration, efficacy, tolerance and adverse effects in a Caucasian population to identify genetic factors predisposing to efficacy and development of adverse drug effects. Furthermore, previous studies have been retrospective. In this chapter, I have used a prospective cohort of patients to study how *CYP2C19* polymorphisms affect the response to CLB. Prospective studies have the advantage as they allow the collection of accurate data on seizure frequency, adverse effects, tolerance and collection of blood for the estimation of CLB and its metabolite at the same time points as the reporting of efficacy or adverse effects. This theoretically should enable better correlation between genotype, concentration of CLB and NDCB, efficacy and adverse effects.

Therefore, the aims of this study were to

1. To estimate the NDCB and CLB concentration in patients recruited in the prospective study.
2. To correlate *CYP 2 C19* genotype to
    a. Concentration of NDCB
    b. Concentration of CLB
    c. Maintenance dose
    d. Efficacy
    e. Adverse effect
    f. Tolerance.
    g. Treatment failure
3. To identify factors affecting efficacy of CLB
4. To identify factors affecting tolerance to CLB
5. To identify factors affecting development of adverse reactions to CLB

5.2. Methods

5.2.1. Study population

This study was a part of the multicentre study – "Pharmacogenetics of GABAergic mechanisms of benefit and harm in epilepsy". This study (CLOPS-Clobazam Prospective cohort Study) is a prospective multicentre study involving an established network of clinicians collaborating in multicentre clinical trials (Marson, et al., 2007). The inclusion criteria of the patients were:

Patients with continued seizures despite current treatment with between 1 and 3 conventional AEDs (carbamazepine, valproate, lamotrigine, phenytoin, topiramate, levetiracetam, gabapentin). Patients who may have been treated previously with AEDs not in this list will also be included.

a. Patients have been on their existing combination of AEDs for at least 3 months.
b. Addition of CLB to existing AED regime or switch of one of current AEDs for CLB is clinically indicated.
c. Any epilepsy syndrome (generalized, localization-related, unclassified).

The exclusion criteria of the patients were:

d. Significant learning disability
e. Patient and/or relatives unable to maintain a seizure diary.

Information on age, sex, ethnic group, seizure frequency over the previous 3 months, seizure types, aetiology of epilepsy, AEDs taken at time CLB started, number of AEDs tried before CLB, learning difficulty, and neurological signs was collected, when the decision was made to administer CLB. The data on other AEDs taken were

used for analysis involving co-medications; data on neurological signs was used to make a diagnosis of patients.

Patients were recruited from different centres for this prospective cohort study. A blood sample was taken at recruitment for DNA. Efficacy was defined as at least 50% reduction in seizure frequency when compared to baseline seizure frequency, after 3 months of CLB treatment. The recruiting physician recorded baseline seizure frequency, after consulting the patients on the occurrence of seizures in the 3 months prior to treatment with CLB. Since the patients maintained diaries, the information provided by them can be trusted. Responders were defined as patients who achieved 50% reduction in seizure frequency after 3 months of CLB treatment, when compared to baseline seizure frequency (seizure frequency before starting CLB). The definition of tolerance has been controversial. Loscher and Schmidt (2006) have defined tolerance as any conversion from a responsive status (more than 50% seizure reduction) to a non responsive status. For this study, if the seizure frequency of a patient was reduced to 50% at any time during the 12 month follow up period and later on increased to above 50%, that patient was considered tolerant to CLB.

Patients entered the cohort on the date that the decision to add CLB is made, with time set to zero for analyses when the first CLB tablet was taken. All the patients, except those who stopped CLB treatment, were followed up for 12 months. Outcomes included **(1)Time to withdrawal of CLB** (a) for any reason (b) due to side effects (c) due to inadequate seizure control and (d) combination of b and c. The information gathered from above (a-d) was used to ascertain the relationship between treatment failure and genotype. (2) **Seizure control** - Seizure frequency per month at 3, 6, 9, and 12 months was compared to baseline seizure frequency. (3) **Adverse**

effects -This was assessed using the Liverpool Adverse Events Profile which was completed by patients at baseline, 3, 6, 9 and 12 months (Baker, et al., 1995). For my thesis, only drowsiness, dizziness and weight gain were analysed. If any patients developed any of these adverse effects, during the 12 month follow up period, they were included in the analysis.

Patients were followed up at 3, 6, 9 and 12 months for data on seizure frequency and drug withdrawal data, AED treatment, blood for CLB levels and the Liverpool Adverse Events Profile. The last CLB dose taken by patients was either on the previous night (20.00-22.00 hrs) or in the morning (7:00- 9.00) of blood collection. Blood samples were collected in the morning or afternoon. The time at which the blood samples were taken was not recorded. These blood samples were used to ascertain the concentrations of CLB, NDCB and NDCB/CLB ratio and to check compliance. NDCB/CLB ratio was used as a phenotypic indicator of CYP2C19 activity.

5.2.2. Measurement of clobazam (CLB) & N-desmethlylclobazam (NDCB) levels

The concentration of CLB and NDCB was measured using the methods described in chapter 4.

5.2.3. Selection of SNPs in the *CYP2C19* gene

SNPs were simultaneously selected from public databases and websites such as CYP450 allele nomenclature website (http://www.cypalleles.ki.se/), dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez), UCSC (http://genome.ucsc.edu/), Ensemble (http://www.ensembl.org/index.html) and HapMap (http://www.hapmap.org/), with the help of SNP prediction software programmes

PupaSNP (Conde, et al., 2004) and *FastSNP* (http://fastsnp.ibms.sinica.edu.tw/pages/input_CandidateGeneSearch.jsp). The characteristics of the SNPs were: (a) location of the SNP (exons, promoter region, exon - intron boundary, 3'untranslated region, 5' UTR), (b) minor allele frequency more than 5%, (c) population (Caucasian), (d) presence of SNPs in conserved regions of the gene (Loots, et al., 2000) and (e) function (kindly refer to chapter 2).

The potential effects of these SNPs were explored using software programs Polyphen (http://genetics.bwh.harvard.edu/pph/) & SIFT (http://www.blocks.fhcrc.org/sift/SIFT.html). Both these programs predict possible effects of an amino acid substitution on protein function. Polyphen can also predict the effect of an amino acid substitution on protein structure. In an attempt not to omit any deleterious SNPs and to represent most or all common variation, I utilised the tagging SNP strategy using HapMap data to select SNPs. HapMap contains high density SNP data that covers the entire genome of population of Europe, African and Asian ancestry. Linkage Disequilibrium (LD) can be quantified using Haploview. This allows us to generate tagging SNPs which predict the rest of the SNPs in a gene or across the genome. The SNP data on *CYP2C19* genes were downloaded from the HapMap website. Haploview (r^2 = 0.8, 2kb upstream of the gene) was used to identify tagging SNPs in *CYP2C19* genes (http://www.broadinstitute.org/haploview/haploview-downloads#DOWNLOAD). A literature search was also performed to identify any SNPs that were reported to affect the function of CYP2C19. The nonsynonymous SNPs identified from all of the above websites and programs were examined by Polyphen and SIFT to predict the effect of any amino acid substitution on protein structure. A final list of SNPs selected for

genotyping was based on the selection criteria mentioned earlier, with emphasis on its known functional role or potential to alter the activity of CYP2C19.

5.2.4. Genotyping

All samples were genotyped using the TaqMan allelic discrimination assays according to the manufacturer's instructions on the ABI PRISM 7900HT Fast Real-Time PCR System platform. Genotyping was done without knowledge of the clinical data or the concentrations of CLB and NDCB. All of the reactions were done in the presence of negative controls. 10% of the samples (positive controls) for each SNP were genotyped twice and checked for any discrepancies. These positive controls were randomly selected for each allele of a SNP. Each 5µl reaction contained 2.5µl of TaqMan® genotyping master mix, 0.25µl of TaqMan® pre-designed SNP genotyping assay, 1µl of DNA and 1.25µl of water. The conditions used for the PCR were 50°C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 sec and 60 °C for 1 min.

5.2.5. Statistical Analyses

Descriptive statistics were used to describe the demographic characteristics of the patients participating in this study - age, sex, type of epilepsy, weight and co-medications. Chi-square tests (for comparisons of categorical data) were used to compare the demographic characteristics, pharmacokinetic and pharmacodynamic data among the *CYP2C19* genotypes. Correlation between dose and (a) concentration of CLB, (b) concentration of NDCB, and (c) the ratio of NDCB/CLB concentration was estimated using the Pearsons correlation coefficient. If an association was found between the ratio and more than one of these factors, a multiple regression analysis was planned.

The correlations between efficacy, tolerance and adverse drug effects, and predictors were explored. The predictors tested were genotype, epilepsy type, NDCB/CLB concentration, and baseline seizure frequency. Chi-square test was used to compare the distribution of genotypes and epilepsy types and unpaired t-test was used to compare the baseline frequency and NDCB/CLB concentration among:

1. Responders and non responders
2. Patients who developed tolerance and those who did not
3. The patients who developed adverse drug reactions and those who did not.

The parameters used to measure efficacy of CLB were:

1. Seizure frequency reduction to 50% after 3 months of CLB treatment
2. Seizure freedom at 3 months.

A logistic regression was performed including the variables significantly associated with efficacy, tolerance and adverse drug reaction. Kaplan-Meier analysis was done to estimate whether there is a significant difference in the mean time to treatment failure to occur among the various genotypes of SNPs- rs4244285, rs12248560 and rs11568732. The analyses which involved multiple comparisons were corrected by Bonferroni's correction.

5.3. Results

5.3.1. Study population

This prospective study included 120 Caucasian patients with epilepsy. The mean age and body weight were 40.48 ± 1.19 (range: 18–73 years) and 75.94 ± 2.31 kg (range: 40–130.0 kg), respectively. In total, 9 patients (7.5%) had generalized seizures and 104 patients (86.67%) had partial seizures (Table 5.1). Body weight was available in only 67 patients.

Table 5.1. Demographic characteristics of patients in this study

Demographic characteristics	Values
Age (years)	
Mean	40.48±1.19
Range	18-73
Males	61
Females	59
Body weight (Kg)	
Mean	75.94±2.31
Range	40-130
Epilepsy type	
Partial epilepsy	104
Generalised epilepsy	9
Unclassified	7

71.6% of patients were on inducer or inhibitor antiepileptic co-medications, which included inducers such as carbamazepine, phenytoin, phenobarbitone, primidone,

oxcarbazepine and topiramate. Valproic acid (VPA) was the only CYP450 inhibitory antiepileptic drug co-administered to these patients.

5.3.2. Selection of putative functional SNPs in *CYP2C19*

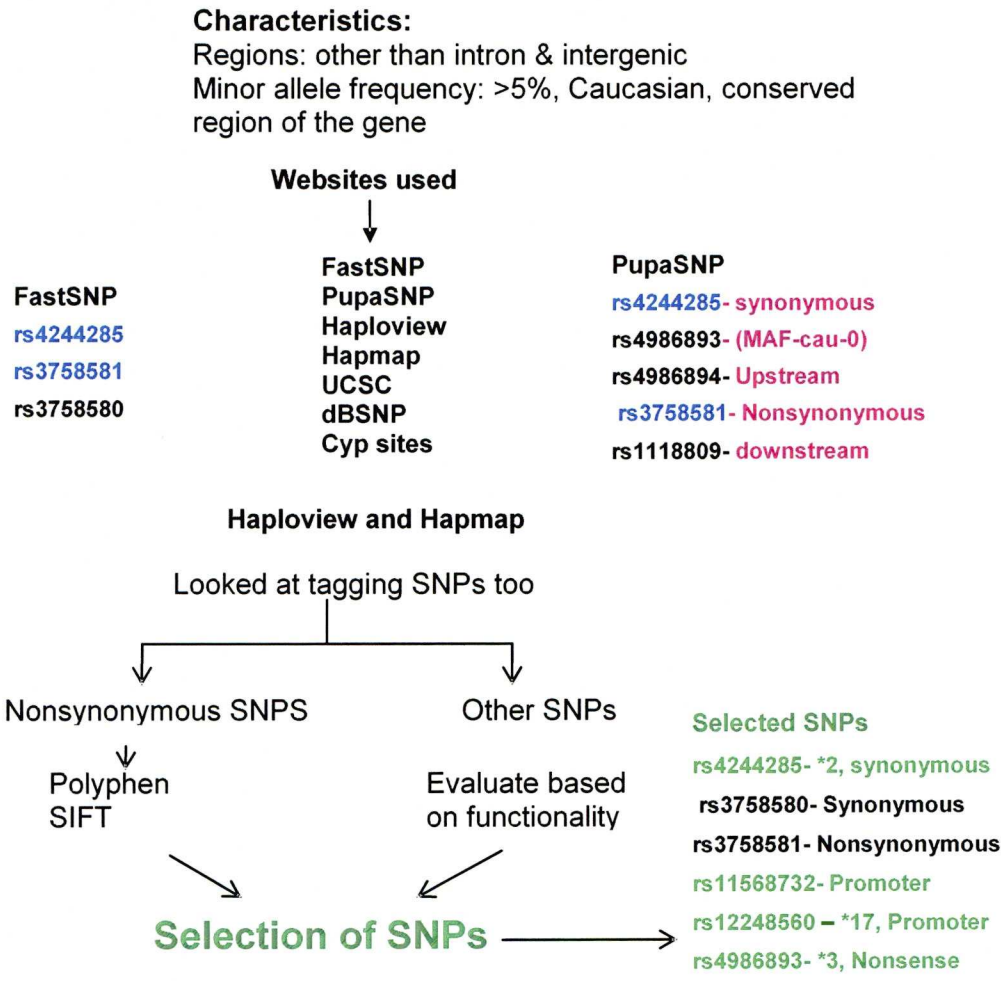


Figure 5.2. Shows the summary of the selection criteria used with the list of SNPs selected to genotype (colured in green). SNPs selected by more than one program is shown in blue.

The proven functional SNPs in the *CYP2C19* gene from the P450 allele nomenclature web site are shown in Table 5.2. Only 3 SNPs had a minor allele frequency (MAF) above 5%. These 3 SNPs (rs4244285, rs12248560 and rs3758581) were considered for genotyping. The number of patients to be recruited for this study was 400. The

MAF of SNPs should be at least 5% to be detected in this number of patients. In addition, the lower the MAF of SNPs, the larger number of SNPs required to represent the genetic variation across the genomic region. The CYP2C19 *3 polymorphism (rs4986893) was also considered for genotyping taking into consideration its functional importance as mentioned in the introduction.

Table 5.2. Proven functional SNPs in *CYP2C19* from *CYP2C19* allele nomenclature web site (compiled from http://www.ncbi.nlm.nih.gov/snp/; http://www.cypalleles.ki.se/cyp2c19.htm).

Allele name -*	**rs number**	**Change in DNA**	**Amino acid change**	**MAF in Caucasian population**	**Effect on CYP2C19 Activity**
1					
2& 21	**rs4244285** **	G681A (40b-DEL 643-682)		**0.15**	Decreases
3	**rs4986893** **	G636A	W212X	**0-0.50**	Decreases
4	rs28399504	A1G	M1V	0.01	Nil (*in vitro*)
5	rs56337013	C1297T	R433W	no data	
6		G395A	R132Q	0.01	Nil (*in vitro*)
7		19294T>A		0	Splicing defect
8	rs41291556	(T358C transition in exon 3)	W120R	0.01	Decreased (*in vitro*)
9	rs17884712	108296G.A	R120H	0.01	Decreased (*in vitro*)
10	rs6413438	C101927T	P227L	0	Decreased (*in vitro*)
11		G108278A	R150H	0.03	Nil (*in vitro*)
12		A30871C	Stop491Cys	f=0	No holoprotein (*in vitro*)
13			R410C	f=0	Unaltered activity (*in vitro*)
14		T20829C	L17P	f=0	
15		A120824C	I19L	f= 0	
16		30871A.C	R442C	not known	Japanese patients
17	**rs12248560** **	806C>T and 3402C>T		**0 .22**	Increases
18& 19	rs**3758581,**** other SNPs	A991G		0	Deleterious
20	rs4986893	G636A	W212X	0	Decreases
22	**rs3758581**, other SNPs	A991G	I331V	**0.051**	Deleterious
23	**rs3758581,** other SNPs	A991G	I331V	**0.051**	Deleterious

Highlighted markers=higher frequency; ** SNPs considered for further selection

Tagging SNPs that cover genetic variation in the whole gene were selected by tagging SNP strategy, using HapMap data as shown in Table 5.3. The LD map of *CYP2C19* is shown in Figure 5.3. rs4244285, rs3758580, rs10509676, rs4532967 and rs4986894 were in complete linkage disequilibrium (LD). rs4244285 is better known as CYP2C19*2 and is associated with decreased catalytic activity of the enzyme (De Morais, et al., 1994). Therefore rs4244285 was selected. rs3758581 and rs11568732 are in LD, while rs3758580 and rs3758581 are very close together making it difficult to genotype. Hence rs11568732 was given preference. rs12248560 is a promoter SNP which increases the activity of CYP2C19 with a minor allele frequency of 21.8%. The rest of the tagging SNPs were in the introns, which was one of our exclusion criteria. In addition, I used software programs FastSNP and PupaSNP to predict putative functional SNPs. The results are given in Figure 5.4. The final selection of SNPs was thus made based on the selection criteria, giving credence particularly to their functional implications (Figure 5.4).

Table 5.3. Tagging SNPs in *CYP2C19* identified by Haploview using Hapmap data.

rs number	Contig Position	dbSNP allele	Function – Region.	MAF in Caucasian population	Functional importance
rs4244285	15290142	G/A	Synonymous – exon 5	0.15	(40b-DEL 643-682) disrupts splicing site,
rs10509676	15271571	A/T	Intron 1		
rs3758580	15351148	C/T	Synonymous - exon 7	0.15	
rs4532967				0.15	
rs4986894	15270891	C/T	5' near gene	0.16	
rs3758581	15351149	G	Missense- exon 7	0.05	
rs11568732	15270100	G/T	5' near gene	0.06	
rs12248560	15270183	C/T	5' near gene	0.22	CYP2C19 *17, increases the activity of CYP2C19.
rs4917623	15358094	C/T	Intron 7	0.49	
rs7916649	15283110	A/G	Intron 1	0 .55	
rs4388808	15284582	A/G	Intron 3	0.19	
rs4362080	15285915	A/G	Intron 3	0.78	
rs7088784	15289899	A/G	Intron 4	0.08	
rs1322179	15323768	A/G	Intron 5	0.08	
rs10786172	15329620	A/G	Intron 6	0.36	

Highlighted SNPs were included in the list of SNPs to genotype

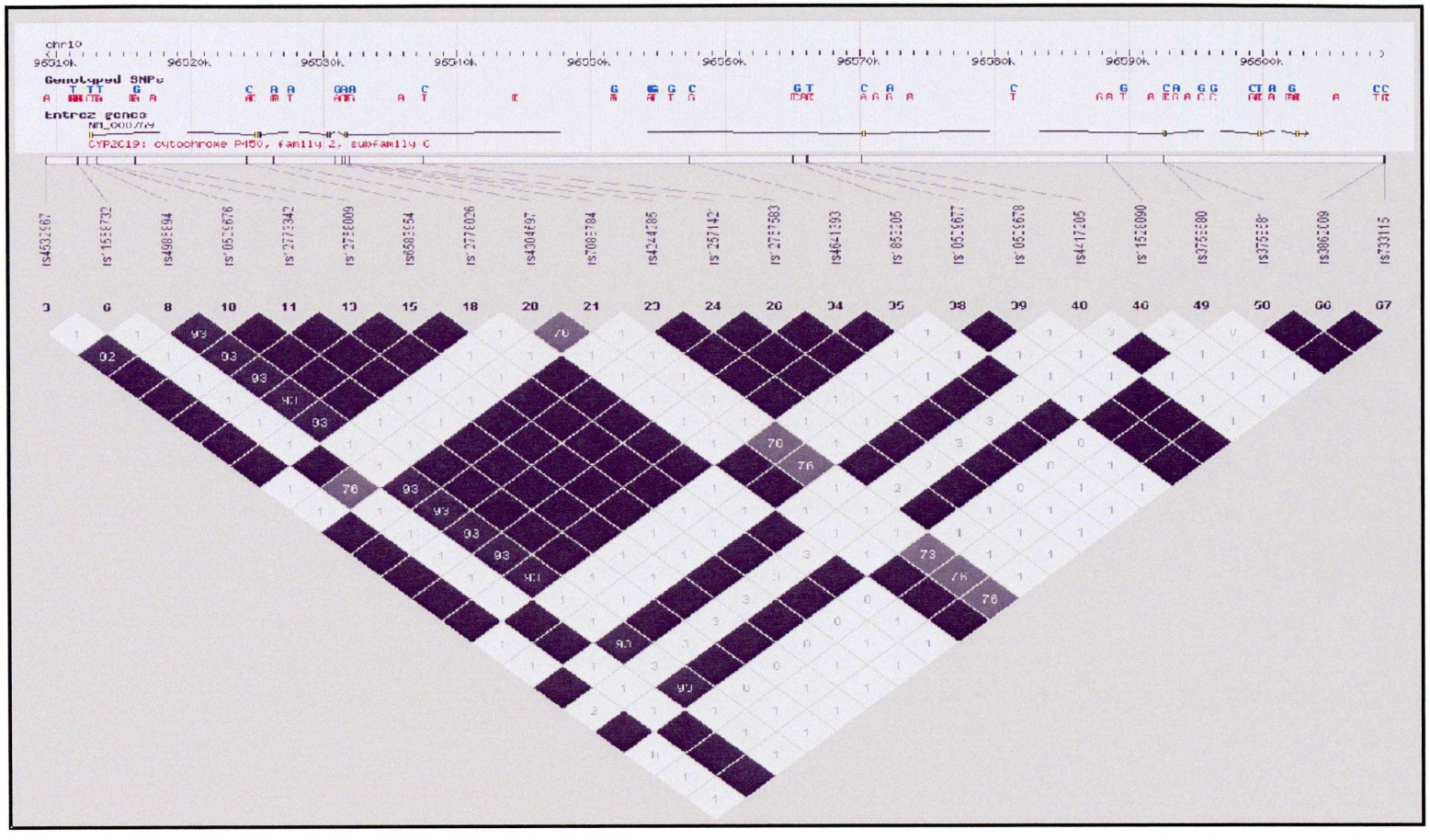


Figure 5.3. shows linkage disequilibrium (LD) of *CYP2C19* gene. The depth of the black colour indicates the strength of the pairwise LD, varying from deep black for full LD (D'=1) to white for no LD existing (D'=0). The picture was produced using HapMap database and the HAPLOVIEW software.

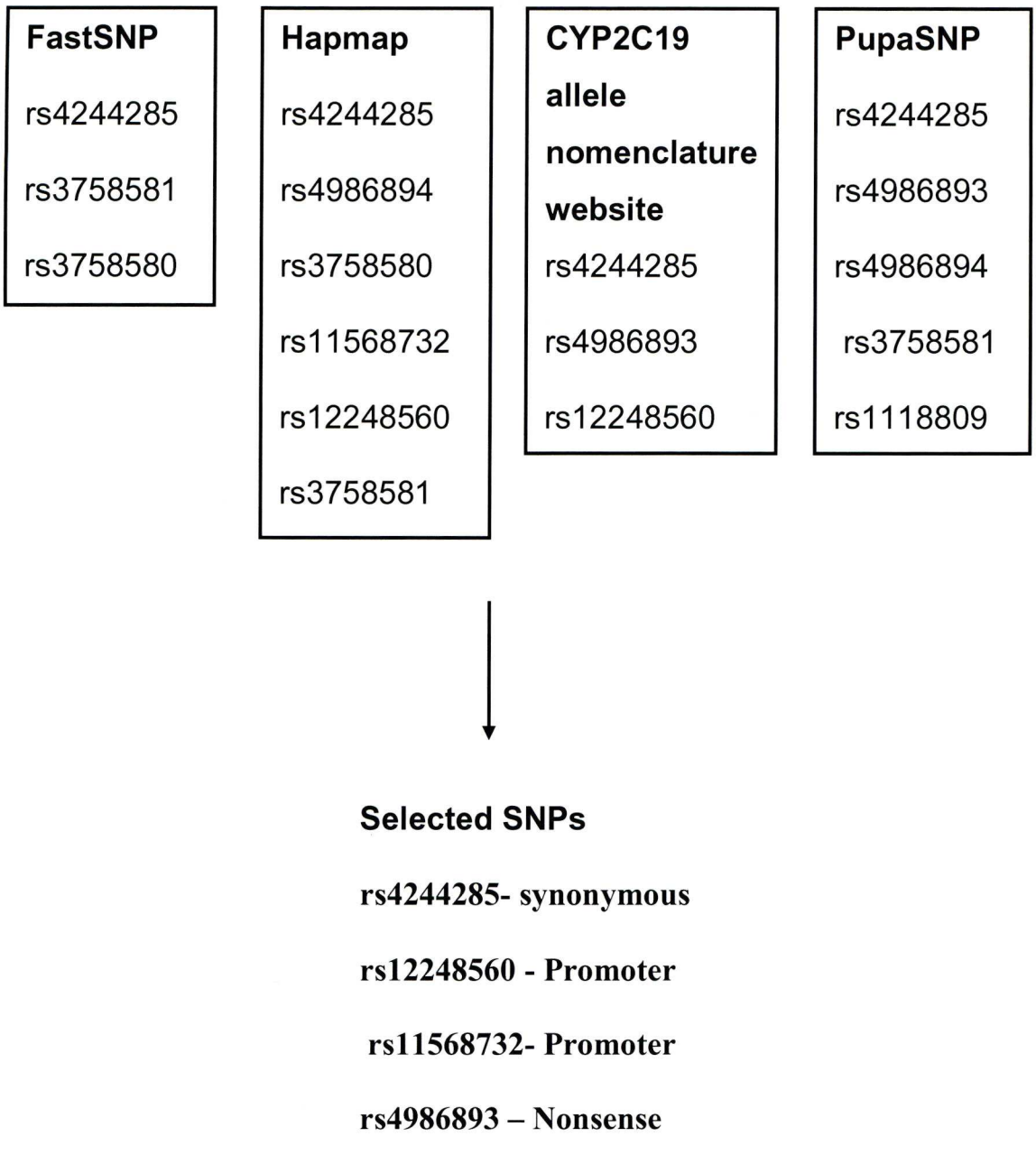


Figure 5.4. Showing the SNPs selected by various programmes and the final selection of SNPs

5.3.3. Allele frequencies of the selected SNPs

Allelic frequency of the selected SNPs was ascertained by real time PCR. The table below (5.4) shows the allelic frequencies in our study population. All the SNPs were in H-W equilibrium. The allele frequencies were comparable to those in dbSNP as proved by chi square test (Table 5.5). 5 patients were not genotyped either due to inadequate quantity of blood collected or poor quality of DNA.

Table 5.4. Allelic frequency of genotyped SNPs in *CYP2C19*

SNP genotyped	No. of individuals	Minor allele frequency	Major allele frequency	dbSNP Minor allele
rs4244285	115	A: 0.15	G: 0.85	0.15
rs12248560	115	T: 0.25	C: 0.75	0.22
rs11568732	115	C: 0.08	A: 0.92	0.06
rs4986893	115	G: 0.00	A:1.00	0.00

Patients with the homozygous major allele, heterozygous and homozygous minor alleles for rs4244285 (CYP2C19*2) were classified as homozygous extensive metabolisers (EMs), heterozygous EMs and poor metabolizers (PMs), respectively according to convention. For SNPs rs12448560 and rs11568732, the patients were classified according to the total count of mutant allele(s): group 0, homozygous major allele; group 1, heterozygous allele; group 2, homozygous minor allele. Since all the patients genotyped for rs4986893 belonged to group 0, this SNP was not considered for further analysis.

Table 5.5. Shows the SNPs in Hardy - Weinberg equilibrium (as shown by the X^2 test)

SNP id	Genotype	Observed No. of people	Expected No. of people	X^2	*p value*
rs4244285	G G	84	83		
	G A	27	30	0.004	0.948
	A A	4	3		
rs12248560	C C	65	63		
	T C	44	42	0.000	1
	T T	6	7		
rs11568732	A A	98	98		
	A C	16	17	0.004	0.948
	C C	1	1		

The demographic characteristics of patients did not differ significantly when stratified for the genotypes represented by the SNPs rs4244285 (Table 5.6). A significantly larger percentage of patients with the homozygous minor allele (rs12248560) received VPA (Table 5.7). However the numbers were small and it is likely that it is a chance finding. There were more homozygous major males; more heterozygous females, with an overall balance of the sexes (Table 5.8).

Table 5.6. Demographic characteristics of patients stratified by genotypes at SNP rs4244285

Parameters	Homozygous Major allele (n=85)	Heterozygous (n=26)	Homozygous Minor allele (n=4)	*p* value
Age				
Mean	41.54 ± 1.45	39 ± 2.46	34.25 ± 4.82	0.4
Males	42 (49.4%)	14 (53.8%)	3 (75%)	0.6
Females	43 (50.6%)	12 (46.2%)	1 (25%)	
Body weight	74.15 ±2.64	82.13±6.01	70.50±13.50	0.4
Epilepsy type				
Partial epilepsy	73 (85.9%)	23 (88.5%)	4 (100%)	0.2
Generalised epilepsy	9 (10.6%)	0	0	
Unclassified	3 (3.5%)	3 (12%)	0	
Co-medications				
Inducers	54 (63.5%)	18 (69.2%)	2 (50%)	0.7
Inhibitors	15 (17.6%)	1 (3.8%)	1 (25%)	0.2

Data is represented as mean ± standard error of the mean (SEM) or as number of patients (percentage)
** Body weight was available in only 67 patients. n= number of patients

Table 5.7. Demographic characteristics of patients stratified by genotypes at SNP rs12248560

Parameters	Homozygous Major allele (CC) (n=64)	Heterozygous (CT) (n=45)	Homozygous Minor allele (TT) (n=6)	*p* value
Mean	41.63±1.71	39.51±1.79	40±6.65	
Males	37(57.8%)	18 (40%)	4 (66.6%)	0.14
Females	27 (42.2%)	27 (60%)	2 (33.3%)	
Body weight (Kg)	38	28	1	0.4
Mean	78±3.24	72.31±3.67	92	
Partial epilepsy	54 (84.4%)	41 (91.1%)	5 (83.3%)	0.7
Generalised epilepsy	5 (7.8%)	3 (6.6%)	1 (16.7%)	
Unclassified	5 (7.8%)	1 (2.2%)	0	
Co-medications				
Inducers	40 (62.5%)	29 (64.4%)	5 (83.3%)	0.6
Inhibitors	5 (7.8%)	10 (22.2%)	2 (33.3%)	**0.05**

Data is represented as mean ± standard error of mean (SEM) or as number of patients (percentage)

** Body weight of only 67 patients were available, n= number of patients, Significant *p* values highlighted

Table 5.8. Demographic characteristics of patients stratified by genotypes at rs11568732

Parameters	Homozygous Major allele (AA) (n=101)	Heterozygous AC (n=13)	Homozygous Minor allele CC (n=1)	*p* value
Age				
Mean ± SE	40.28 ± 1.27	43.69 ± 4.52	46	0.6
Gender				
Males	56 (54.5 %)	3 (23.1%)	0	
Females	45 (44.6%)	10 (76.9 %)	1 (100%)	0.053
Body weight in Kg (mean ± SEM)**	59 (76.92 ± 2.71)	7 (68 ± 1.69)	1 (66)	
Epilepsy type				
Partial epilepsy	88 (87.1%)	11 (84.6%)	1 (100%)	
Generalised epilepsy	7 (6.9%)	2 (15.4%)	0	0.9
Unclassified	6 (5.9%)	0	0	
Co-medications				
Inducers	65 (64.4%)	9 (69.2%)	0	0.4
Inhibitors	16 (15.8%)	1 (7.7%)	0	0.7

Data is represented as mean ± standard error of mean (SEM) or as number of patients (percentage)
** Body weight of only 67 patients were available, n= number of patients.

The concentrations of CLB and NDCB were measured in 94 patients, after 3 months of CLB treatment. At the time of 6th month blood analysis, only 72 patients had

completed 6 months of CLB treatment. Of these 72 patients, 3rd month CLB estimation was not done for 11 patients. The reasons include blood samples arriving very late and insufficient blood to extract plasma. In total, the concentration of CLB and NDCB was estimated in 120 patients. Seventeen patients were excluded from analysis involving correlation of genotype of *CYP2C19* to the pharmacokinetics of CLB and NDCB, due to the following reasons: Genotype was not done in 5 patients, CLB was not detected in 5 patients, 2 patients had interfering peaks and one patient refused to give blood. In the case of 2 patients, there was insufficient blood to extract plasma. Two patients were on diazepam, and so could not be included as diazepam was used as the ISfor the estimation of CLB and NDCB. The mean (of 3rd month and 6th month) of CLB dose, CLB concentration, NDCB concentration and NDCB/CLB ratio were not significantly different between the 3rd and the 6th month of follow up (Table 5.9). Therefore the mean of the 3rd and 6th month, of CLB dose, CLB concentration, NDCB concentration and NDCB/CLB ratio was used in further analyses. In cases where the mean was not available, the 3rd or 6th month value (of CLB dose, CLB concentration, NDCB concentration and NDCB/CLB) was used for analysis since there was no significant difference between the 3rd, 6th month and mean of the two values.

Table 5.9. Showing the comparison of pharmacokinetic parameters of clobazam

Characteristic	3rd Month, n=94	6th Month, n=72	Mean of 3rd & 6th month, n=60	*p value*
Clobazam dose (mg)	15.4±0.61	17.71±0.82	16.02±0.62	0.22 (3 m Vs 6 m) 0.73 (3 m Vs Mean) 0.44 (6 m Vs Mean)
Clobazam concentration	161.9± 10.89	194.38± 16.90	185.6± 15.45	0.27 (3 m Vs 6 m) 0.73 (3 m Vs Mean) 1.00 (6 m Vs Mean)
NDCB concentration	997.5± 114.23	1145.99± 122.44	1189.96± 148.84	1.00 (3 m Vs 6 m) 0.87 (3 m Vs Mean) 1.00 (6 m Vs Mean)
NDCB/CLB concentration	8.8±1.1	8.84±1.08	8.54±1.08	1.00 (3 m Vs 6 m) 1.00 (3 m Vs Mean) 1.00 (6 m Vs Mean)

Data is represented as mean ± standard error of mean (SEM), n= number of patients
p values after Bonferroni correction for multiple testing. Vs = versus

5.3.4. Association between *CYP2C19* genotypes & clobazam pharmacokinetics

The rs4244285 (CYP2C19*2) SNP genotype was associated with NDCB concentrations, the concentration/dose (C/D) ratio of NDCB and the NDCB/CLB concentration ratio, but there was no association with the CLB daily dose (Table 5.10). The homozygous EMs, heterozygous EMs, and PM genotypes had a greater impact on the concentration of NDCB than on CLB. The NDCB concentration in PMs was 5.75 and 4.48 times higher than in homozygous EMs and

heterozygotes respectively. In addition, the EMs had a significantly lower value of NDCB/CLB ratio than heterozygotes and PMs (Table 5.10).

Table 5.10. Association between the CYP2C19*2 (rs4244285) genotypes and clobazam pharmacokinetics

Parameters	Groups	n	Mean	*p value*
CLB/CLB mg (ng/ml/mg)	Homozygous major allele (0)	79	10.86±1.04	1(2 Vs 0)
	Heterozygous (1)	21	10.67±1.01	1 (1 Vs 0)
	Homozygous minor allele (2)	3	9.30±3.59	1 (2 Vs 1)
NDCB/ CLB mg(ng/ml/mg)	Homozygous major allele (0)	79	51.71±4.31	**0.00** (2 Vs 0)
	Heterozygous (1)	21	63.08±7.01	0.71 (1 Vs 0)
	Homozygous minor allele (2)	3	304.30±86.03	**0.00** (2 Vs 1)
CLB Dose	Homozygous major allele (0)	83	16.62±0.66	1 (2 Vs 0)
	Heterozygous (1)	22	16.02±1.00	1(1 Vs 0)
	Homozygous minor allele (2)	3	18.33±1.67	1 (2 Vs 1)
NDCB/CLB ratio	Homozygous major allele (0)	79	8.27±1.09	**0.00** (2 Vs 0)
	Heterozygous (1)	21	7.56±1.22	1 (1 Vs 0)
	Homozygous minor allele (2)	3	37.00±7.39	**0.00** (2 Vs 1)
NDCB concentration (ng/ml)	Homozygous major allele (0)	79	835.08±73.90	**0.00** (2 Vs 0)
	Heterozygous (1)	21	1072.21±154.79	0.54 (1 Vs 0)
	Homozygous minor allele (2)	3	4798.67±1129.36	**0.00** (2 Vs 1)
CLB concentration (ng/ml)	Homozygous major allele (0)	79	167.35±13.67	1 (2 Vs 0)
	Heterozygous (1)	21	172.89±17.53	1(1 Vs 0)
	Homozygous minor allele (2)	3	151.91±52.38	1 (2 Vs 1)

p value (significance across groups). Significant *p value*s are highlighted. Data expressed as mean ± standard error of mean (SEM). n= number of patients. Vs= versus

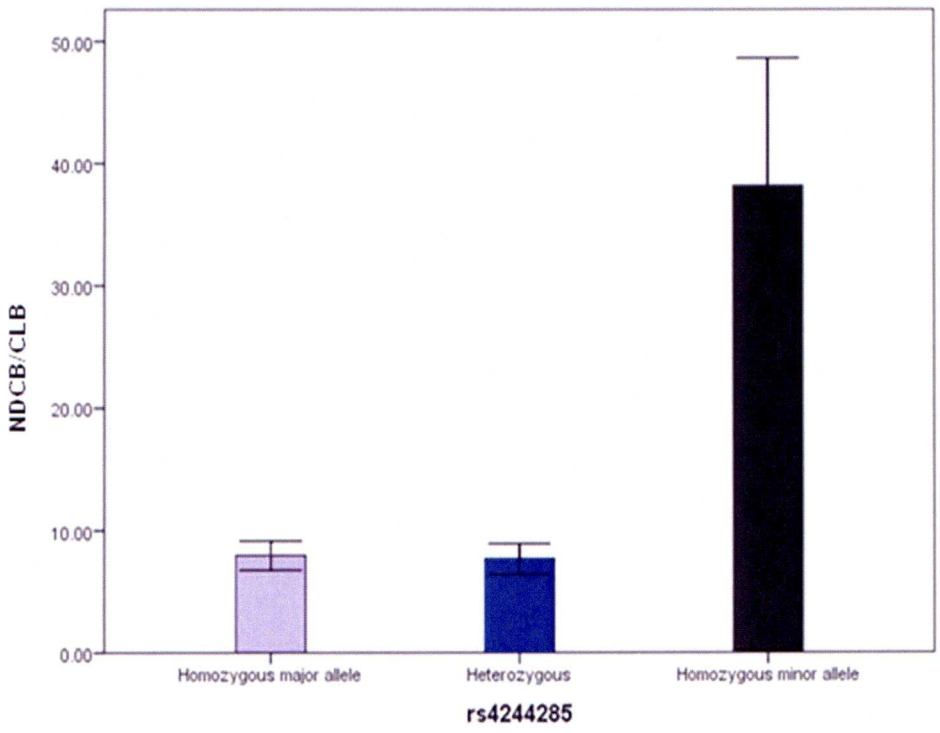


Figure 5.5. Shows the association of the CYP2C19*2 genotypes (rs4244285) with the NDCB/CLB ratio. Each bar represents the mean ± standard error of mean (SEM) of each genotype. This is the result from 103 patients.

The CYP2C19*17 (rs12248560) genotype was associated with the CLB dose. Patients who were homozygous for the minor allele were on higher CLB doses than heterozygous or homozygous major allele patients (*p value* = 0.086 and 0.012, respectively). Other pharmacokinetic parameters, mentioned in the table below, were not influenced by rs12248560 (Table 5.11).

Table 5.11. Association between CYP2C19*17 rs12248560 genotypes and clobazam and NDCB concentrations

Parameter	Genotype	n	Mean	*p* value*
CLB concentration (ng/ml)	Homozygous major allele (0)	55	172.38±14.98	0.230 (2 Vs 0)
	Heterozygous (1)	42	174.03±18.28	1 (1 Vs 0)
	Homozygous minor allele (2)	6	86.20±22.29	0.227 (2 Vs 1)
NDCB concentration (ng/ml)	Homozygous major allele (0)	55	1159.03±158.46	1 (2 Vs 0)
	Heterozygous (1)	42	800.79±101.47	0.222 (1 Vs 0)
	Homozygous minor allele (2)	6	917.38±224.94	1 (2 Vs 1)
NDCB/CLB ratio	Homozygous major allele (0)	55	9.81±1.56	0.956 (2 Vs 0)
	Heterozygous (1)	42	7.09±1.29	0.588 (1 Vs 0)
	Homozygous minor allele (2)	6	14.20±3.29	0.339 (2 Vs 1)
Dose of CLB (mg)	Homozygous major allele (0)	64	15.54±.63	**0.012** (2 Vs 0)
	Heterozygous (1)	45	17.14±.95	0.459 (1 Vs 0)
	Homozygous minor allele (2)	6	22.50±2.81	0.086 (2 Vs 1)

*Significant *p values* are highlighted. Data expressed as mean ± standard error of mean (SEM). n= number of patients. Vs= versus

rs11568732 had an impact on NDCB concentrations. The concentration of NDCB was 1049.89± 109.42, 693.14 ± 94.16 and 432.24 ng/ml in groups 0, 1 and 2 respectively (Table 5.12). Since there was only one patient in group 2, a post hoc test was not done after ANOVA. ANOVA alone did not show any significant

difference between the groups. However, when group 2 was excluded and a t-test done, there was a significant difference in the concentration of NDCB between groups 0 and 1. In addition, the mean ratio of NDCB/CLB concentration was 8.87 ± 0.99, 10.08 ± 4.38 and 2.18 in groups 0, 1, and 2. ANOVA alone did not show any significant difference between the groups. A t-test performed after excluding group 2 did not show a significant difference between the means of the NDCB/CLB ratio in groups 0 and 1.

Table 5.12. Association between rs11567832 genotypes and clobazam pharmacokinetics

Parameter	Genotype	n	Mean	*p value*
CLB concentration (ng/ml)	Homozygous major allele (0)	89	168.00± 12.38	
	Heterozygous (1)	13	165.6329± 24.98	0.955
	Homozygous minor allele (2)	1	201.63	
NDCB concentration (ng/ml)	Homozygous major allele (0)	89	1049.89± 109.42	
	Heterozygous (1)	13	693.14 ± 94.16	**0.017** [1]
	Homozygous minor allele (2)	1	432.24	
NDCB/CLB ratio	Homozygous major allele (0)	89	8.87 ± 0.99	
	Heterozygous (1)	13	10.08 ± 4.38	0.745
	Homozygous minor allele (2)	1	2.18	
CLB dose (mg)	Homozygous major allele (0)	94	16.70± 0.60	
	Heterozygous (1)	13	15.96 ± 1.43	0.472
	Homozygous minor allele (2)	1	10	

Significant *p value*s are highlighted. Data expressed as mean ± standard error of mean (SEM). 1- group 2 was excluded and a t-test done. n = number of patients

The concentration of CLB was significantly lower in patients who were on enzyme inducers (*p* value = 0.003) (Table 5.13). In addition, the dose of CLB was higher in patients on inducers, but this was not significant. Co-medication with VPA, an enzyme inhibitor, did not affect any pharmacokinetic parameters.

Table 5.13. The distribution of pharmacokinetic parameters in patients who had inducers as co-medications (1) compared with patients who were not on these co-medications (0)

Parameter	Status of Co-medications	n	Mean	*p* value
CLB concentration (ng/ml)	0	42	221.38 ±22.83	**0.003**
	1	68	143.29±11.09	
NDCB concentration (ng/ml)	0	42	990.82±192.67	0.9
	1	69	1009.46±93.61	
NDCB/CLB ratio	0	42	6.47±1.29	0.06
	1	66	9.80±1.156	
CLB dose (mg)	0	44	14.71±0.81	0.1
	1	76	16.77±0.85	

Significant *p value* is highlighted. Data expressed as mean ± standard error of mean (SEM). n= number of patients

The mean concentration of CLB or NDCB did not correlate with the mean dose of CLB using Pearson's correlation coefficient. Multiple regression was used to identify predictors of the NDCB/CLB ratio. The variables tested were genotype (rs4242285, rs12248560, and rs11568732), dose of CLB and co-medications.

rs4242285 was the only predictive factor identified. The details of the model obtained are shown in Table 5.14.

Table 5.14. The details of the multiple regression done for NDCB/CLB ratio

Parameters of multiple regression	Values
R square	0.100
F	11.280
P (using the stepwise method).	0.00
Predictor Variable	rs4244285; 0=GG; 1=GA; 2=AA
Beta	5.654
P value	0.001

5.3.5. Association between *CYP2C19* genotypes and the efficacy of CLB

A 50% or greater reduction in the seizure frequency when compared to the baseline before starting CLB therapy (responder status) was achieved in 55 (46.6%) patients, while 17 (14.4%) of the total patients (118, see below) achieved seizure freedom. The mean CLB daily doses in cases with 50% reduction in seizures (responders) and nonresponders were 15.86 ± 0.79 and 16.35 ± 0.95 respectively ($p = 0.7$). Table 5.15 shows the responder rates for each genotype for rs4244285. Two subjects were excluded from the analyses because their baseline seizure frequency was not known

and 5 patients were not genotyped. The reasons were inadequate quantity of blood or poor quality DNA.

The responders were more likely to possess the mutant allele(s) than the nonresponders. All the patients who were PMs had 50% reduction in their seizures when compared to baseline seizure frequency. It is notable that 50% of the PMs achieved seizure freedom while only 10% of the homozygous EMs obtained seizure freedom, with 27% of the heterozygotes achieving seizure freedom. However the numbers are small. To summarise, the efficacy (seizure freedom and 50% reduction in seizure frequency) was greater in the PMs than in heterozygous EMs who demonstrated better efficacy than homozygous EMs.

Table 5.15. Efficacy of clobazam amongst the rs4244285 genotypes

Efficacy Characteristic	Yes	No	*P* value
50% reduction in seizures			
Homozygous (83)	32 (38.55%)	51 (61.45%)	**0.005**
Heterozygous (26)	17 (65.38%)	9 (34.62%)	
Homozygous minor allele (4)	4 (100%)	0	
Seizure freedom			
Homozygous (83)	8 (9.64%)	75 (90.36%)	**0.014**
Heterozygous (26)	7 (26.92%)	19 (73.08%)	
Homozygous minor allele (4)	2 (50%)	2 (50%)	

Significant *p value* is highlighted. Data expressed as number of patients (percentage)

The SNPs rs12248560 and rs11568732 did not affect the efficacy of CLB. There was no significant difference in the number of patients who had 50% or greater reduction in seizure frequency among the different genotypes for rs12248560 (p=0.499). Seizure freedom at 3 months of treatment also showed a similar pattern (p =0.634). The efficacy of CLB (50% reduction in seizure frequency (p=0.464) and seizure freedom (p=0.914) was not associated with the different genotypes of rs11568732.

Logistic regression was used to predict seizure freedom from epilepsy type, genotype (rs4244285, rs12248560, and rs11568732), baseline seizure frequency, dose of CLB, concentration of CLB and NDCB, ratio of NDCB/CLB. Only rs4244285 was a significant predictor of seizure freedom. For every mutant allele of rs4244285, the odds of seizure freedom (versus presence of seizures) increased by a factor of 4.16 (Table 5.16 and 5.17).

Table 5.16. Shows the parameters of the logistic regression model for seizure freedom

Variables in the equation [c]							
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Odds ratio**
Step 1a	rs4244285	1.42	0.48	8.88	1	0.01	4.16
	Constant	-2.07	0.36	32.47	1	1.21	0.13
Step 2b	rs4244285	1.72	0.54	10.35	1	0.00	5.61
	Epilepsy_type			5.05	2	0.08	
	Epilepsy_type(d)	0.10	1.25	0.007	1	0.93	1.12
	Epilepsy_type(e)	2.02	1.48	1.87	1	0.17	7.54
	Constant	-2.53	1.28	3.89	1	0.04	0.08

a. Variable(s) entered on step 1: rs4244285.
b. Variable(s) entered on step 2: epilepsy_type,d = partial epilepsy, e= generalised epilepsy,
c. Stepwise procedure stopped because removing the least significant variable result in a previously fitted model. B= regression coefficient

Table 5.17. Shows the parameters of the logistic regression model for seizure freedom

Omnibus Tests of Model Coefficients				
		Chi-square	**df**	**Sig.**
Step 1	Step	9.30	1	0.002
	Block	9.30	1	0.002
	Model	9.30	1	0.002
Step 2	Step	4.46	2	0.107
	Block	13.77	3	0.003
	Model	13.77	3	0.003
Hosmer and Lemeshow Test				
Step		Chi-square	df	Sig.
1		0	0	3.5E+308
2		0.140	3	0.987

A logistic regression model was used to predict 50% or greater reduction in seizure frequency from epilepsy type, genotype (rs4244285, rs12248560, and rs11568732), baseline seizure frequency, dose of CLB, concentration of CLB and NDCB, and ratio of NDCB/CLB. Only rs4244285 was a significant predictor of 50% or greater seizure reduction. For every mutant allele in rs4244285, the odds of 50% or greater seizure reduction (versus non-reduction) increased by a factor of 3.8 (Table 5.18 and 5.19).

Table 5.18. Shows the parameters of the logistic regression of 50% seizure reduction

Variables in the equation		B	S.E.	Wald	Df	Sig.	Odds ratio
Step 1a	rs4244285	1.33	0.49	7.15	1	0.007	3.78
	Constant	-0.35	0.23	2.18	1	0.139	0.70
Step 2b	rs4244285	1.41	0.50	7.84	1	0.005	4.11
	rs11568732	1.27	0.69	3.42	1	0.064	3.59
	Constant	-0.52	0.27	4.17	1	0.041	0.59

a. Variable(s) entered on step 1: rs4244285.
b. Variable(s) entered on step 2: rs11568732, B= regression coefficient

Table 5.19. Showing the parameters of the logistic regression of 50% seizure reduction

Omnibus Tests of Model Coefficients		Chi-square	Df	Sig.
Step 1	Step	8.80	1	0.003
	Block	8.80	1	0.003
	Model	8.80	1	0.003
Step 2	Step	4.11	1	0.042
	Block	12.92	2	0.002
	Model	12.92	2	0.002

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig.
1	0	0	3.48+308
2	0.946	2	0.62

A Kaplan-Meier survival analysis was undertaken to explore the differences in cumulative incidence of treatment failure in the genotypes of SNPs rs4244285, rs12248560 and rs11568732. Twenty five patients experienced treatment failure. 12 patients had inadequate seizure control, 2 patients had inadequate seizure control and adverse effects, and 13 patients had unacceptable adverse effects to CLB. There was no significant difference in the estimated mean survival time (treatment failure) among the genotypes of these SNPs.

5.3.6. Association between *CYP2C19* genotypes and tolerance to CLB

Baseline seizure data or seizure data of the 3rd, 6th, 9th or 12th month weren't available for 56 patients. Sixteen patients (33.3%) out of the remaining 48 patients developed tolerance to CLB therapy. The incidence of tolerance did not differ among the genotypes of any of the SNPs (rs4244285, rs12248560 and rs11568732) tested. There was a significant difference ($p = 0.023$) in mean CLB daily doses in subjects who developed tolerance than in those who did not (19.68 ± 1.06 Vs 16.51 ± 0.80 respectively). The NDCB /CLB ratio was significantly higher in patients who developed tolerance (13.51 ± 2.68) than in patients who still responded to CLB (7.05 ± 1.04) (*p value* = 0.038). The NDCB concentration was higher in patients who developed tolerance but this failed to reach significance (*p value* =0.055) (Table 5.20). In the 12 month follow up period after CLB was started, 5 (31.2%) out of 16 patients did not respond to CLB once tolerance developed. The seizure data after tolerance developed were not available for 4 patients. 43.75% (7/16) developed tolerance by the time of the last follow up. Further data on seizure frequency were not available.

A logistic regression model was used to predict tolerance from epilepsy type, genotype (rs4244285, rs12248560, and rs11568732), dose of CLB, concentration of CLB and NDCB, ratio of NDCB/CLB. However none of these parameters were identified as predictors for developing tolerance.

Table 5.20. Showing the distribution of pharmacokinetic parameters in patients who developed tolerance (1) and patients who did not develop tolerance (0).

Pharmacokinetic Parameters	Degree of tolerance	n	Mean	*P value*
CLB concentration	0	48	186.70 ± 20.40	0.376
	1	16	157.38 ± 25.49	
NDCB concentration	0	48	929.12 ±121.09	0.055
	1	16	1553.16 ± 425.03	
NDCB/CLB ratio	0	46	7.06 ± 1.04	**0.038**
	1	16	13.52 ± 2.69	
Dose of CLB	0	48	16.51 ± 0.81	**0.023**
	1	16	19.69 ± 1.07	

Mean is expressed as mean ± standard error of mean (SEM). Significant *p value* is highlighted. n= number of patients

5.3.7. Association between *CYP2C19* genotypes & adverse effects to CLB

For this thesis, the incidence of only drowsiness, weight gain and dizziness was evaluated. The total incidence of adverse effects was 42.5%. The most common adverse effect, experienced alone or in combination with others, was weight gain (23.3%), followed by drowsiness (15%) and dizziness (14.1%). Only SNP rs11568732 was associated with adverse effects (Table 5.21). The incidence of

dizziness decreased with the mutant allele(s) for rs11568732 (p =0.043). However, weight gain and drowsiness were not affected by this SNP. Only one patient had homozygous minor allele and did not develop any adverse effects. This finding has to be replicated in a larger study group to prove that this is not a chance finding. The incidence of these adverse effects did not differ among the rs4244285 and rs12248560 genotypes (Table 5.22 and 5.23).

Table 5.21. Association between rs11568732 genotypes and adverse reactions

Adverse reaction	Homozygous Major allele (0) (n = 101)	Heterozygous (1) (n=13)	Homozygous minor allele (2) (n=1)	*p*-value
Drowsiness	15 (14.85%)	2 (15.38%)	0	0.9
Dizziness	15 (14.85%)	1 (7.69%)	0	**0.04**
Weight gain	24 (23.76%)	3 (23.08%)	0	0.9

Significant *p* values highlighted; n= number of patients

Table 5.22. Association between rs4244285 genotypes and adverse reactions

Adverse reaction	Homozygous EMS (0) (n=85)	Heterozygous EMS (1) (n=26)	Homozygous minor allele (2) (n=4)	*p*-value
Drowsiness	13 (15.29%)	3 (15.54%)	1 (25%)	0.8
Dizziness	12 (14.12%)	4 (15.38%)	1 (25%)	0.8
Weight gain	20 (23.53%)	7 (26.92%)	0	0.5

n= number of patients

Table 5.23. Association between rs12248560 genotypes and adverse reactions

Adverse reaction	Homozygous Major allele (0) (n =64)	Heterozygous (1) (n =45)	Homozygous minor allele (2) (n=6)	*p*-value
Drowsiness	10 (15.63%)	6 (13.33%)	1 (16.67%)	0.9
Dizziness	10 (15.63%)	6 (13.33%)	1 (16.67%)	0.9
Weight gain	15 (23.43%)	11 (24.4%)	1 (16.67%)	0.9

n= number of patients

5.4. Discussion

To the best of my knowledge, this is the first prospective study conducted in Caucasians exploring the impact of *CYP2C19* polymorphisms on the metabolism, efficacy and adverse effects of CLB. An attempt has also been made to define the covariates or predictors that influence the metabolism, tolerance and propensity to develop adverse drug effects with CLB. I have documented an association between CYP2C19*2 polymorphism and (a) NDCB concentration (b) NDCB/CLB dose and (c) NDCB/CLB concentration. In addition, CYP2C19*2 had an impact on the efficacy of CLB. However CYP2C19*2 did not influence the development of tolerance and adverse drug effects to CLB. By contrast, rs12248560 (CYP2C19*17) influenced the CLB dose in patients ($p = 0.012$), while rs11568732 was associated with the development of dizziness. A multiple regression model for the ratio of NDCB/CLB showed that CYP2C19*2 genotype was the only factor influencing this ratio. A logistic regression model for efficacy also predicted the same. In summary, I

present novel data which show that *CYP2C19* SNPs have an impact on the metabolism, efficacy and toxicity of CLB.

The selection of SNPs for genotyping is very important in candidate gene studies. I feel that the criteria we used for SNP selection was optimal. I wanted to genotype a set of SNPs, which capture the genetic variation in the *CYP2C19* gene and at the same time produce an effect that has sufficient impact to be picked up in our study. The minor allele frequency of the SNPs should be high enough for this (at least 5%). Saito et al (2007) used a tagging SNP strategy and selected most of the SNPs (5/6) that I considered (Table 5.3), to represent the 6 haplotypes of *CYP2C19* gene. Therefore our genotyping strategy is likely to have been successful in covering the genetic variation across the whole gene.

Our study population had Caucasian ancestry. This is important since heterogeneous populations have different genotype frequencies, which might lead to spurious results in genotype-phenotype correlations. All the subjects in this study were adults. The Concentration/Dose (C/D) ratio of CLB in children was significantly lower than adults, among the patients who were homozygous for the major allele of SNP rs4244285, leading to doubtful results (Seo, et al., 2008). In our study, the demographic characteristics were comparable among the genotypes for all our SNPs (Tables 5.6-5.8).

In our study, two blood samples were collected: one at month 3 and the second at month 6 after starting CLB treatment. There was no significant difference between the 3rd, 6th and the mean of the two (NDCB/CLB concentration, NDCB concentration, CLB concentration and CLB dose) (Table 5.9). For all the further

analysis, we used the mean (of 3rd and 6th month) of these parameters, where available.

The NDCB concentration, C/D of NDCB and NDCB/CLB concentration increased as the rs4244285 minor allele increased. However, the only significant difference was detected between the PMs and (a) heterozygous EMs and (b) homozygous EMs (p =0.00). All the previous studies had demonstrated a significant difference in NDCB/CLB ratio among all 3 groups (Contin, et al., 2002; Kosaki, et al., 2004; Parmeggiani, et al., 2004), or between homozygous EMs and heterozygous EMs (Giraud, et al., 2004). However the concentration of NDCB was much higher than CLB as reported in earlier studies. There was no significant difference in CLB concentration across various genotypes of rs4244285, similar to recent studies (Kosaki, et al., 2004; Seo, et al., 2008).

Patients with rs12248560 homozygous minor allele had significantly higher dose of CLB (22.50mg) than the homozygous major allele patients (14.80mg) (Table 5.10). SNP rs12248560 increases the activity of CYP2C19. Theoretically patients with homozygous minor allele should have a higher dose of CLB, which is evident in our study. The concentration of CLB was insignificantly lower in homozygous mutant allele group (group 2). In addition the concentration of NDCB is not significantly higher in group 0 when compared to homozygous minor allele (group 2). Probably a larger sample size might be able to confirm these findings.

There was only one patient with homozygous minor allele (group 2) of SNP rs11568732. The patients in group 1 had a significantly lower concentration of NDCB and C/D of NDCB than group 0. Functional studies on this SNP haven't been done. However it is only 83 bp (base pairs) upstream of rs12248560, which induces

gene expression in mouse. In humans, rs12248560 is responsible for ultra rapid metabolism of drugs metabolized by CYP2C19. This has been associated with lack of response to some proton pump inhibitors and anti depressants, due to an ultra rapid clearance of these drugs (Sim, et al., 2006; Saito, et al., 2007). The hepatic reporter expression by Sim et al (2006), to assess the effect of rs12248560 on *CYP2C19* included the region of SNP rs11568732. Therefore this SNP too could increase the expression of *CYP2C19*. However the frequency of this SNP is only 6.1% (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=11568732) in Caucasian population while that of rs12248560 is 21.6% (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12248560). Functional assays should be conducted to verify the function of rs11568732. If rs11568732 increases the activity of CYP2C19, the concentration of NDCB will be lower in subjects with this mutant allele. Though only one patient had homozygous minor allele, the NDCB concentration was 2.4 fold lower than group 0 and 1.6 fold lower than group1 patients (Table 5.12). It is interesting to note that the only patient with minor allele did not have a mutant allele of CYP2C19*2 (rs4244285). In addition the ratio of NDCB/CLB concentration was more than 4 fold lower in the patients in group 2 when compared to group 0. This adds further weight to our assumption that rs11568732 could increase the activity of CYP2C19. However, conclusions cannot be drawn from the findings in a single patient. Genotypes had a lesser influence on CLB concentration. This could be due to the fact that CLB is metabolised to NDCB by CYP2B6, CYP3A4 and CYP2C19 while NDCB is converted to 4'-hydroxynorclobazam primarily by CYP2C19 (Giraud, et al., 2004).

46.6% of the subjects experienced reduction in seizures by 50% when compared to that of the baseline. This is consistent with various studies including a multi centre

study in Canada (Canadian Clobazam Cooperative Group, 1991). It is interesting to note that all the patients with homozygous minor allele (group 2) of rs4244285 responded to CLB. In addition, 65.4% patients in the heterozygous group had 50% reduction in seizure frequency compared to base line. This was higher than the responders in the homozygous major allele group. This is consistent with other studies (Seo, et al., 2008).

44.9% (44/98), 53.8% (7/13) and 100% (1/1) patients of group 0, 1 and 2 of rs11568732, experienced 50% reduction in seizure frequency $p = 0.464$. A larger sample size with more patients with homozygous minor allele will be able to clearly prove the association of rs11568732 and efficacy. rs12248560 did not affect the efficacy of CLB.

15.04% of the study population attained seizure freedom. Only rs4244285 was associated with seizure freedom. The number of patients who achieved seizure freedom in PMs (50%) was significantly higher than heterozygous EMs (26.92%) and homozygous EMs (9.64%). The number of patients with seizure freedom increased with the number of mutant alleles. The dose of CLB did not significantly differ between the responders (patients who had more than 50% seizure reduction) and non responders, contrary to the findings of Seo et al (2008). SNPs rs11568732 and rs12248560 did not affect this parameter.

Tolerance is a major drawback of CLB, the frequency of which varies widely (Shorvon, 1998; Remy, 2007). This is hindering the wide spread prescription of this relatively safe drug. 33.33% of the patients developed tolerance in this study. There was no significant difference in the number of patients who developed tolerance among the different genotypes of SNPs – rs4244285, in concordance with previous

results (Seo, et al., 2008). rs11568732 and rs12248560 too did not influence the development of tolerance.

For my thesis only drowsiness, weight gain and dizziness were evaluated, since these were the common adverse effects reported in this study and in other studies. 42.5% of patients developed adverse drug effects. 23.3% complained of weight gain, while 15% and 14.1% patients developed drowsiness and dizziness respectively. Only SNP rs11568732 influenced the development of adverse drug effects. Though there was only one patient with homozygous mutant allele, this patient did not develop drowsiness, dizziness or weight gain. The patients who complained of dizziness were significantly lower in group 1 than in group 0. There was no significant difference in CLB dose between the genotypes of rs11568732. However the concentration of NDCB was significantly lower (p = 0.02) in heterozygous patients than in patients with homozygous major allele. If as mentioned earlier, rs11568732 increases the activity of CYP2C19, the probability of adverse drug effects occurring in heterozygous and homozygous minor allele patient groups will be lower when compared to the homozygous major allele group patients as seen in this study. These findings and hypotheses are of modest value, until replicated in a larger cohort of patients.

Though a prospective study, this study has some drawbacks. Patient data including weight, epilepsy type and baseline seizure frequency were not available for all patients (for details kindly refer to respective result sections). Some patients have missed their follow up (3 patients the 3rd month follow up and 4 patients the 6th month follow up). So blood for CLB and NDCB concentration estimation was not obtained on their 3rd month or 6th month follow up.

To summarise, I present here a prospective cohort study evaluating the role of CYP2C19 polymorphisms in the metabolism, efficacy and development of adverse effects of CLB. In addition I have investigated the factors affecting these parameters. This could be the first step towards developing a genetic test which predicts an individual's predisposition to respond well to CLB or develop adverse effects. This will help clinicians use this relatively safe drug judiciously, thus leading to personalised medicine benefiting the patient and reducing the costs of health care.

Chapter 6

General discussion

Epilepsy is one of the most common neurological disorders, affecting 0.7% of the UK population (Gao, et al., 2008). It is associated with significant mortality, morbidity and psychosocial distress. Despite the fact that around 20 drugs are available on the market today, almost 30% of the patients do not respond to any of these drugs or drug combinations (Dalby, 2004). These patients are therefore treated on a 'trial and error basis' initially by up-titration of the dosage, and subsequently by addition of other drugs such that the patient ends up on multiple AEDs in an effort to gain seizure control.

VGB and CLB are drugs with some efficacy in these refractory patients (Gram, et al., 1985; Koeppen, et al., 1987; Munn, et al., 1988; Guberman, et al., 1990; Guberman and Bruni, 2000; Michael and Marson, 2008). VGB is particularly effective in paediatric patients with West syndrome and tuberous sclerosis (Antoniuk and Bruck, 1996; Herranz and Argumosa, 2000; Wheless, et al., 2007). However, VGB produces VFD in almost 40% of patients (Wheless, et al., 2007). CLB, though a relatively safe drug, causes tolerance in a large proportion of patients. Side effects have been reported in 20-85% of patients in clinical trials. However, only 5-15% of these lead to a change in dose or termination of treatment (Koeppen, et al., 1987; Canadian Clobazam Cooperative Group, 1991). Drowsiness, dizziness, headache, nausea and weight gain are listed as the most common adverse drug effects (Allen, et al., 1983). These factors may deter clinicians from prescribing CLB (Dalby, 2004).

The aim of the thesis was to test the hypothesis that genetic factors may be important in determining individual variability in response to these AEDs. Specifically for VGB, my focus was on patients who developed VVFD, and to test the hypothesis

that GABA-T, the enzyme irreversibly inhibited by VGB, may be implicated in the pathogenesis of the variability. For CLB, identification of genetic variation in patients who (1) vary in their response to CLB; (2) who develop tolerance; and (3) who develop adverse drug effects would facilitate the stratification of prescribing of CLB to maximise its effectiveness.

In chapter 2, the aim was to define the phenotype-genotype relationship for the GABA-T enzyme. Identifying functional SNPs which are clinically relevant among the 12 million SNPs in the human genome is similar to searching for a 'needle in a haystack' (http://www.ncbi.nlm.nih.gov/projects/SNP/). Therefore utmost importance was given to selection criteria of SNPs. I predicted putative functional SNPs from websites like dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez), Ensemble (http://www.ensembl.org/index.html) and UCSC (http://genome.ucsc.edu/) using software programs like PupaSNP (Conde, et al., 2004) and SNPper (Riva and Kohane, 2004). By standardising and validating an assay, I was able to evaluate the function of GABA-T in platelets. Given the non-invasive nature of my approach, and the need to control for confounding factors, I first tested for the functional effects of SNPs using healthy volunteers. Though my sample size was small (n=32), an association between rs1731017 and platelet GABA-T activity was identified. This is a novel finding.

Interestingly, however, Kinirons et al. (2006) failed to find an association between this common SNP (minor allele frequency 40%) and VVFD. This illustrates an important point. Targeting certain SNPs may not define a functional deficit if other factors (genetic or non-genetic) are more important. In order to further investigate this, platelet GABA-T activity was assessed in patients with VVFD in comparison to

patients who had been on VGB without the development of VVFD. Interestingly, platelet GABA-T activity was significantly lower in patients with VVFD than in patients with normal vision (chapter 3), a highly unexpected finding given that only 14 patients were studied. The explanation for this is unknown, but may suggest the presence of rare variants which have a marked effect on activity, or that other genetic factors (beyond the ABAT gene) can influence its activity. Alternatively, epigenetic factors may also be important. These hypotheses need further testing – we have recently initiated a deep re-sequencing project for the ABAT gene in order to identify novel, and rare, variants. Clearly any finding from this will need to be subject to replication in a larger cohort of patients, and functional analysis of any mutants identified. However since VGB is given to only refractory epilepsy patients (Eke, et al., 1997; James, et al., 2009), it might be difficult to get a large cohort of patients. An alternative (and additional) approach, which is also being undertaken as part of the overall research programme, is to perform a genome-wide association scan. Since the mechanism of VVFD is unknown, this will allow an unbiased assessment of possible genetic variants on a genome scale, including CNV.

CLB is a relatively idiosyncratic drug, favored by some epileptologists, but perhaps ignored by the majority. This may partly be related to its modest efficacy in the overall population in which it is used, and the development of tolerance. The polymorphic enzyme CYP2C19 is involved in the metabolism of CLB to its major active metabolite NDCB. In order to assess the importance of the metabolism of CLB in determining its response characteristics, I developed a reverse phase HPLC method (Chapter 4) which had high sensitivity and specificity. This allowed the estimation of the plasma concentrations of CLB and its major metabolite NDCB in a prospective study setting, which has never been undertaken previously. These

kinetic parameters were then related to polymorphisms in the *CYP2C19* gene. *CYP2C19* is well known to be polymorphically expressed, and was initially identified through variability in the metabolism of mephenytoin, and was thus initially known as mephenytoin hydroxylase. There are many known substrates for CYP2C19 including CLB. The most intensively studied SNPs in *CYP2C19* include those which cause loss of function (CYP2C19*2, and CYP2C19*3), and those which cause a gain of function (CYP2C19*17). The patients in my cohort were genotyped for all of these polymorphisms (chapter 5). I did not identify any patients who were carrying the *3 allele, which was expected given the low population frequency of this allele in the Caucasian population. I found an association between the CYP2C19*2 polymorphism and (1) the metabolism of CLB and (2) its efficacy. Prior studies have reported similar results in relation to the kinetic parameters (Kosaki, et al., 2004; Seo, et al., 2008), and in relation to efficacy (Seo, et al., 2008). By contrast, rs11568732 was associated with CLB-induced dizziness, which was significantly lower in patients with variant alleles when compared to those with two major alleles. Comparison of the homozygous major allele group and heterozygous patients showed that the former had significantly higher concentration of NDCB. This was done because there was only one patient in homozygous minor allele group. Patients who were homozygous for the minor allele of CYP2C19*17 were on a significantly higher dose of CLB than the heterozygotes or carriers of two major alleles. The effect of these SNPs - rs12248560 and rs11568732 - has not previously been investigated in relation to CLB. These are novel findings, but which nevertheless need to be replicated in another patient cohort before being assessed for clinical validity and clinical utility.

There is a long road to travel before genetic tests for CLB are available for routine clinical use. It has been stated that pharmacogenetic tests for drugs with a narrow therapeutic index take a shorter time to be incorporated in clinical practice (Nebert and Vesell, 2006). Clinical guidelines have been published for the use of pharmacogenetic tests in the prescription of drugs with a narrow therapeutic index like warfarin, tricyclic antidepressants and risperidone (Voora, et al., 2005; Hillman, et al., 2005; de Leon, 2007). However, CLB has a wide therapeutic index and the adverse drug effects associated with its use are mild. However, it can cause tolerance and there seem to be sub-groups of patients who seem to respond well to CLB. Thus, it can still be argued that there is a need to stratify patients for CLB treatment, but the method by which this will be done, and whether it will be effective, needs further investigation.

Many high penetrance, predominantly monogenic disorders in drug metabolism were reported between 1940 and 1990. It was predicted that personalized medicines were just around the corner. However, the human genome project revealed the complexity of the human genome (Nebert, et al., 2008). In addition, many pharmacogenetic studies could not be replicated. Examples include the association of serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients (Masellis, et al., 1998; Joober, et al., 1999) and the effects of apolipoprotein E genotype on clinical response to tacrine (Farlow, et al., 1996; Rigaud, et al., 2000). Now it is recognized that the concerted effort of all the major players - pharmaceutical industry/analytical industry, regulatory bodies, scientists, clinicians and patients groups - will be essential to implement pharmacogenetic tests on a routine basis in clinics (Swen, et al., 2007). Since it has been proposed that many genetic variants of small to moderate effects predispose or cause a complex

disease or a complex trait, like efficacy or toxicity of a drug, there is a need to (a) ensure that phenotyping strategies are as accurate as possible, and (b) to collect large sample sizes which will only be possible through multi-centre collaborations. Many GWAS studies in complex diseases have now been completed. Although these have shown many new loci, the effect sizes have been quite low (RR 1.2-1.7) leading to pessimism that these variants may never be used as genomic biomarkers (McCarthy, et al., 2008). However, the importance of these studies has been to highlight novel pathways which are likely to lead to new drug targets. By contrast, genome wide studies have produced some striking findings even with small sample sizes indicating that pharmacogenomic biomarkers may in some cases have much larger effect sizes than those seen in complex diseases (Nieminen, et al., 2008; Daly, et al., 2009). Irrespective of the approach utilized, it is important that studies are of good quality with appropriate reporting standards as recently highlighted (Jorgensen and Williamson, 2008).

The human genome is complex. On many occasions, it is difficult to ascertain either an unequivocal phenotype or genotype. The study of gene transcripts (transcriptomics) and the study of all the proteins that are encoded by the genome (proteomics) may also be important in delivering personalized medicines. The major limitation of these branches of science is the source of tissue for analysis (blood, excreta (e.g. urine and faeces)) and tissue that contains the relevant cDNA (e.g. surgical biopsy of tumour, other tissue biopsies, placenta and foreskin). Metabolomics, the study of metabolite profiling in 'multicellular systems' may also help in predicting the drug response in individuals. All these branches of science - pharmacogenomics, transcriptomics, proteomics and metabonomics - working together will complement each other and ultimately lead to personalized medicine

approaches in clinical practice (Nebert and Vesell, 2006). In accordance with this, the findings from this thesis provide some interesting findings which need further investigation, and support the concept that personalised medicine approaches may be valuable in the future use of VGB and CLB.

Bibliography

Aicardi J, Mumford JP, Dumas C and Wood S (1996) Vigabatrin as initial therapy for infantile spasms: a European retrospective survey. Sabril IS Investigator and Peer Review Groups. *Epilepsia* **37:**638-642.

Allen JW, Oxley J, Robertson MM, Trimble MR, Richens A and Jawad SSM (1983) Clobazam as adjuntive treatment in refractory epilepsy. *BMJ* **286:**1.246-1.247.

Ameen M, Smith CH and Barker JNWN (2002) Pharmacogenetics in clinical dermatology. *Br J Dermatol* **146:**2-6.

Antoniuk SA and Bruck I (1996) West syndrome - Treatment. *Rev Bras Leprol* **32:**43-45.

Aoyagi T, Wada T, Kojima F, Nagai M, Harada S, Takeuchi T and Hirokawa K (1990) Increase in aminobutyrate aminotransferase and cholineacetyltransferase in cerebrum of aged rats. *Chem Pharm Bull (Tokyo)* **38:**1750-1752.

Appleton RE, Peters ACB, Mumford JP and Shaw DE (1999) Randomised, placebo-controlled study of vigabatrin as first-line treatment of infantile spasms. *Epilepsia* **40:**1627-1633.

Arendt RM, Greenblatt DJ, Liebisch DC, Luu MD and Paul SM (1987) Determinants of benzodiazepine brain uptake: lipophilicity versus binding affinity. *Psychopharmacology (Berl)* **93:**72-76.

Armijo JA (1989) 4-aminobutyrate-2-ketoglutarate aminotransferase (GABA-T) in human hair follicle. *Rev Esp Fisiol* **45:**337-338, 339, 340, 341,342.

Armijo JA, Arteaga R, Valiente R and Herranz JL (1989) Platelet GABA-aminotransferase in epileptic patients. *Int J Clin Pharmacol Res* **9:**283-285.

Arteaga R, Herranz JL, Valdizán EM and Armijo JA (1992) Gamma-vinyl GABA (vigabatrin): relationship between dosage, plasma concentrations, platelet GABA-transaminase inhibition, and seizure reduction in epileptic children. *Epilepsia* **33:**923-923.

Arteaga R, Herranz JL and Armijo JA (1993) Platelet GABA-transaminase in epileptic children: influence of epilepsy and anticonvulsants. *Epilepsy Res* **14:**73-85.

Arthur JF, Gardiner EE, Kenny D, Andrews RK and Berndt MC (2008) Platelet receptor redox regulation. *Platelets* **19:**1-8.

Badcock NR and Zoanetti GD (1987) Micro-determination of clobazam and N-desmethylclobazam in plasma or serum by electron-capture gas chromatography. *J Chromatogr* **421:**147-154.

Bagamery K, Kvell K, Barnet M, Landau R and Graham J (2005) Are platelets activated after a rapid, one-step density gradient centrifugation? Evidence from flow cytometric analysis. *Clin Lab Haematol* **27:**75-77.

Baker GA, Jacoby A, Francis P and Chadwick DW (1995) The Liverpool adverse drug events profile. *Epilepsia* **36:**S59.

Bardy AH, Seppala T, Salokorpi T, Granstrom ML and Santavuori P (1991) Monitoring of concentrations of clobazam and norclobazam in serum and saliva of children with epilepsy. *Brain Dev* **13:**174-179.

Barzaghi F, Fournex R and Mantegazza P (1973) Pharmacological and toxicological properties of clobazam (1-phenyl-5-methyl-8-chloro-1,2,4,5-tetrahydro-2,4-diketo-3H-1,5-benzodiazepine), a new psychotherapeutic agent. *Arzneimittelforschung* **23:**683-686.

Bedoya MP, Toledo C, de Vicente M and Gonzalez MP (1988) Comparative study of GABA-T from glial cells, neuronal perikarya cells and synaptosomes. *Comp Biochem Physiol C* **90:**61-64.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA and Mathern GW (2010) Revised terminology and concepts for organization of seizures and epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005–2009. *Epilepsia* **51:**676-685.

Berrettini WH, Umberkoman-Wiita B, Nurnberger JI,Jr, Vogel WH, Gershon ES and Post RM (1982) Platelet GABA-transaminase in affective illness. *Psychiatry Res* **7:**255-260.

Best JL and Acheson JF (2004) The natural history of Vigabatrin associated visual field defects in patients electing to continue their medication. *Eye* **19:**41-44.

Blencowe BJ (2000) Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem Sci* **25:**106-110.

Blume WT, Luders HO, Mizrahi E, Tassinari C, van Emde Boas W, Engel J (2001) Glossary of ictal semiology. *Epilepsia* **42**:1212–1218.

Bolton JB, Rimmer E, Williams J and Richens A (1989) The effect of vigabatrin on brain and platelet GABA-transaminase activities. *Br J Clin Pharmacol* **27 Suppl 1:**35S-42S.

Bowery NG, Price GW, Hudson AL, Hill DR, Wilkin GP and Turnbull MJ (1984) GABA receptor multiplicity. Visualization of different receptor types in the mammalian CNS. *Neuropharmacology* **23:**219-231.

Brachet-Liermain A, Jarry C, Faure O, Guyot M and Loiseau P (1982) Liquid chromatography determination of clobazam and its major metabolite N-desmethylclobazam in human plasma. *Ther Drug Monit* **4:**301.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248-254.

Braestrup C, Nielsen EB, Sonnewald U, Knutsen LJ, Andersen KE, Jansen JA, Frederiksen K, Andersen PH, Mortensen A and Suzdak PD (1990) (R)-N-[4,4-bis(3-methyl-2-thienyl)but-3-en-1-yl]nipecotic acid binds with high affinity to the brain gamma-aminobutyric acid uptake carrier. *J Neurochem* **54:**639-647.

Brockmöller J and Tzvetkov MV (2008) Pharmacogenetics: data, concepts and tools to improve drug discovery and drug treatment. *Eur J Clin Pharmacol* **64:**133-157.

Browne TR, Mattson RH, Penry JK, Smith DB, Treiman DM, Wilder BJ, Ben-Menachem E, Napoliello MJ, Sherry KM and Szabo GK (1987) Vigabatrin for refractory complex partial seizures: multicenter single-blind study with long-term follow-up. *Neurology* **37:**184-189.

Bruni J, Guberman A, Vachon L and Desforges C (2000) Vigabatrin as add-on therapy for adult complex partial seizures: a double-blind, placebo-controlled multicentre study. The Canadian Vigabatrin Study Group. *Seizure* **9:**224-232.

Bun H, Monjanel-Mouterde S, Noel F, Durand A and Cano JP (1990) Effects of age and antiepileptic drugs on plasma levels and kinetics of clobazam and N-desmethylclobazam. *Pharmacol Toxicol* **67:**136-140.

Caccia S, Guiso G and Garattini S (1980) Brain concentrations of clobazam and N-desmethylclobazam and antileptazol activity. *J Pharm Pharmacol* **32:**295-296.

Camacho A and Dimsdale JE (2000) Platelets and psychiatry: lessons learned from old and new studies. *Psychosom Med* **62:**326-336.

Canadian Clobazam Cooperative Group (1991) Clobazam in treatment of refractory epilepsy: the Canadian experience. A retrospective study. *Epilepsia* **32:**407-416.

Canadian Study Group for Childhood Epilepsy (1998) Clobazam has equivalent efficacy to carbamazepine and phenytoin as monotherapy for childhood epilepsy. *Epilepsia* **39:**952-9 5 9.

Carson PE, Flanagan CL, Ickes CE and Alving AS (1956) Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science* **124:**484-485.

Chiron C, Dulac O, Luna D, Palacios L, Mondragon S, Beaumont D and Mumford JP (1990) Vigabatrin in infantile spasms. *Lancet* **335:**363-364.

Chiron C, Dumas C, Jambaqué I, Mumford J and Dulac O (1997) Randomized trial comparing vigabatrin and hydrocortisone in infantile spasms due to tuberous sclerosis. *Epilepsy Res* **26:**389-395.

Clayman CB, Arnold J, Hockwald RS, Yount EH,Jr, Edgcomb JH and Alving AS (1952) Toxicity of primaquine in Caucasians. *JAMA* **149:**1563-1568.

Commission on Classification and Terminology of the International League Against Epilepsy (2010) Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classifiaction and Terminology, 2005–2009. *Epilepsia* **51**:676–685.

Conde L, Vaquerizas JM, Santoyo J, Al-Shahrour F, Ruiz-Llorente S, Robledo M and Dopazo J (2004) PupaSNP Finder: a web tool for finding SNPs with putative effect at transcriptional level. *Nucleic Acids Res* **32:**W242.

Contin M, Sangiorgi S, Riva R, Parmeggiani A, Albani F and Baruzzi A (2002) Evidence of polymorphic CYP2C19 involvement in the human metabolism of N-desmethylclobazam. *Ther Drug Monit* **24:**737-741.

Coppola G (2004) Treatment of partial seizures in childhood: an overview. *CNS drugs* **18:**133-156.

Coppola G, Terraciano AM and Pascotto A (1997) Vigabatrin as add-on therapy in children and adolescents with refractory epilepsy: an open trial. *Brain Dev* **19:**459-463.

Coulter DA (1997) Antiepileptic drug cellular mechanisms of action: where does lamotrigine fit in? *J Child Neurol* **12:**2-9.

Coupland SG, Zackon DH, Leonard BC and Ross TM (2001) Vigabatrin effect on inner retinal function. *Ophthalmology* **108:**1493-6; discussion 1497-8.

Cramer JA, Fisher R, Ben-Menachem E, French J and Mattson RH (1999) New antiepileptic drugs: comparison of key clinical trials. *Epilepsia* **40:**590-600.

Czauderna M and Kowalczyk J (2001) Separation of some mono-, di- and tri-unsaturated fatty acids containing 18 carbon atoms by high-performance liquid chromatography and photodiode array detection. *J Chromatogr B Biomed Sci Appl* **760:**165-178.

Dalby MA (2004) The treatment of epilepsy, in (S. D. Shorvon, E. Perucca, D. Fish and E. Dodson eds) pp 358-364, Blackwell Science, Malden, Mass. ; Oxford.

Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, Daly MJ, Goldstein DB, John S and Nelson MR (2009) HLA-B* 5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet* **41(7):**816-819.

De Biase D, Barra D, Simmaco M, John RA and Bossa F (1995) Primary structure and tissue distribution of human 4-aminobutyrate aminotransferase. *Eur J Biochem* **227:**476-480.

de Leon J (2007) The crucial role of the therapeutic window in understanding the clinical relevance of the poor versus the ultrarapid metabolizer phenotypes in

subjects taking drugs metabolized by CYP2D6 or CYP2C19. *J Clin Psychopharmacol* **27:**241.

De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K and Goldstein JA (1994) Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol Pharmacol* **46:**594-598.

Debler EA and Lajtha A (1987) High-affinity transport of γ-aminobutyric acid, glycine, taurine, L-aspartic acid, and L-glutamic acid in synaptosomal (P2) tissue: a kinetic and substrate specificity analysis. *J Neurochem* **48:**1851-1856.

Dichter MA (2009) Emerging concepts in the pathogenesis of epilepsy and epileptogenesis. *Arch Neurol* **66:**443-447.

Dichter MA and Brodie MJ (1996) New antiepileptic drugs. *N Engl J Med* **334:**1583-1590.

Dinovo EC, Miyada DS and Nakamura RM (1973) *Clin Chem* **19:**994.

Dodson WE (2004) Definitions and classification of epilepsy, in *The treatment of epilepsy* (S. D. Shorvon, E. Perucca, D. Fish and W. E. and Dodson eds) pp 3-20, Blackwell Science, Malden, Mass. ; Oxford.

Dongre VG, Karmuse PP, Rao PP and Kumar A (2008) Development and validation of UPLC method for determination of primaquine phosphate and its impurities. *J Pharm Biomed Anal* **46:**236-242.

Duncan JS (1994) Vigabatrin, in *New Anticonvulsants* (M. R. Trimble ed) pp 113-126, Wiley, Chichester, UK.

Durham SL, Hoke JF and Chen TM (1993) Pharmacokinetics and metabolism of vigabatrin following a single oral dose of [14C] vigabatrin in healthy male volunteers. *Drug Metab Disposition* **21:**480-484.

Dusci L and Hackett L (1987) Simultaneous determination of clobazam, N-desmethyl clobazam and clonazepam in plasma by high performance liquid chromatography. *Ther Drug Monit* **9:**113.

Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K and Beck S (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. *Nat Genet* **38:**1378-1385.

Eke T, Talbot JF and Lawden MC (1997) Severe persistent visual field constriction associated with vigabatrin. *BMJ* **314:**180-181.

Evans DAP, Manley KA and McKusick VA (1960) Genetic control of isoniazid metabolism in man. *BMJ* **2:**485-491.

Farlow MR, Lahiri DK, Poirier J, Davignon J and Hui S (1996) Apolipoprotein E genotype and gender influence response to tacrine therapy. *Ann N Y Acad Sci* **802:**101-110.

FDA U (2001) Guidance for industry: bioanalytical method validation. *US Food and Drug Administration, Rockville, MD*

Feely M and Gibson J (1984) Intermittent clobazam for catamenial epilepsy: Tolerance avoided. *J Neurol Neurosurg Psychiatry* **47:**1279-1282.

Ferrarese C, Sala G, Riva R, Begni B, Zoia C, Tremolizzo L, Galimberti G, Millul A, Bastone A and Mennini T (2001a) Decreased platelet glutamate uptake in patients with amyotrophic lateral sclerosis. *Neurology* **56:**270.

Ferrarese C, Tremolizzo L, Rigoldi M, Sala G, Begni B, Brighina L, Ricci G, Albizzati M, Piolti R and Crosti F (2001b) Decreased platelet glutamate uptake and genetic risk factors in patients with Parkinson's disease. *Neurol Sci* **22:**65-66.

Ferrarese C, Aliprandi A, Tremolizzo L, Stanzani L, De Micheli A, Dolara A and Frattola L (2001c) Increased glutamate in CSF and plasma of patients with HIV dementia. *Neurology* **57:**671-675.

Ferrarese C, Zoia C, Pecora N, Piolti R, Frigo M, Bianchi G, Sala G, Begni B, Riva R and Frattola L (1999) Reduced platelet glutamate uptake in Parkinson's disease. *J Neural Transm* **106:**685-692.

Fielding S and Hoffmann I (1979) Pharmacology of anti-anxiety drugs with special reference to clobazam. *Br J Clin Pharmacol* **7:**7S-15S.

Forsgren L (2004) Epidemiology and prognosis of epilepsy and its treatment, in *The treatment of epilepsy* (S. D. Shorvon, E. Perucca, D. Fish and E. Dodson eds) pp 21-42, Malden, Mass, Blackwell Science, Oxford.

Fox AL (1932) The relationship between chemical constitution and taste. *Proc Natl Acad Sci U S A* **18:**115-120.

Gao S, Juhaeri J and Dai WS (2008) The incidence rate of seizures in relation to BMI in UK adults. *Obesity (Silver Spring)* **16:**2126-2132.

Gardiner SJ and Begg EJ (2006) Pharmacogenetics, Drug-Metabolizing Enzymes, and Clinical Practice. *Pharmacol Rev* **58:**521-590.

Garrod AE (1931) *The inborn factors of disease.* Oxford Univiversity Press, London, UK.

Gastaut H and Low MD (1979) Antiepileptic properties of clobazam, a 1-5 benzodiazepine, in man. *Epilepsia* **20:**437-446.

Gazdzik WR, Podlesny J and Filipek M (1989) HPLC method for simultaneous determination of clobazam and N-desmethylclobazam in human serum, rat serum and rat brain homogenates. *Biomed Chromatogr* **3:**79-81.

Giraud C, Tran A, Rey E, Vincent J, Tréluyer J- and Pons G (2004) In vitro characterization of clobazam metabolism by recombinant cytochrome P450 Enzymes: Importance of CYP2C19. *Drug Metab Dispos* **32:**1279-1286.

Gonnard P, Wicker A, Kouyoumdjian JC and Bloch-Tardy M (1973) Rapid radioisotope method of determination of 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-transaminase) activity. *Biochimie* **55:**509-510.

Gotz E, Feuerstein TJ, Lais A and Meyer DK (1993) Effects of gabapentin on release of gamma-aminobutyric acid from slices of rat neostriatum. *Arzneimittelforschung* **43:**636-638.

Gram L, Klosterskov P and Dam M (1985) gamma-Vinyl GABA: a double-blind placebo-controlled trial in partial epilepsy. *Ann Neurol* **17:**262-266.

Gram L, Larsson OM, Johnsen A and Schousboe A (1989) Experimental studies of the influence of vigabatrin on the GABA system. *Br J Clin Pharmacol* **27 Suppl 1:**13S-17S.

Grant DM, Blum M and Meyer UA (1992) Polymorphisms of N-acetyltransferase genes. *Xenobiotica* **22:**1073-1081.

Greenblatt DJ, Divoll M, Puri SK, Ho I, Zinny MA and Shader RI (1983) Reduced single-dose clearance of clobazam in elderly men predicts increased multiple-dose accumulation. *Clin Pharmacokinet* **8:**83-94.

Greenblatt DJ, Divoll M, Puri SK, Ho I, Zinny MA and Shader RI (1981) Clobazam kinetics in the elderly. *Br J Clin Pharmacol* **12:**631-636.

Gross-Tsur V, Banin E, Shahar E, Shalev RS and Lahat E (2000) Visual impairment in children with epilepsy treated with vigabatrin. *Ann Neurol* **48:**60-64.

Gross-Tsur V, Lahat E, Banin E, Shahar E and Shalev R (2002) Visual field disturbances in epileptic patients treated with vigabatrin (sevril). *Harefuah* **141:**247-249+315.

Guberman A and Bruni J (2000) Long-term open multicentre, add-on trial of vigabatrin in adult resistant partial epilepsy. *Seizure* **9:**112-118.

Guberman A, Couture M, Blaschuk K and Sherwin A (1990) Add-on trial of clobazam in intractable adult epilepsy with plasma level correlations. *Can J Neurol Sci* **17:**311-316.

Haigh JR, Pullar T, Gent JP, Dailley C and Feely M (1987) N-desmethylclobazam: a possible alternative to clobazam in the treatment of refractory epilepsy? *Br J Clin Pharmacol* **23:**213-218.

Hambley JW and Johnston GA (1985) Uptake of gamma-aminobutyric acid by human blood platelets: comparison with CNS uptake. *Life Sci* **36:**2053-2062.

Hardus P, Verduin WM, Postma G, Stilma JS, Berendschot TTJM and Van Veelen CWM (2000a) Concentric contraction of the visual field in patients with temporal lobe epilepsy and its association with the use of vigabatrin medication. *Epilepsia* **41:**581-587.

Hardus P, Verduin WM, Postma G, Stilma JS, Berendschot TTJM and Van Veelen CWM (2000b) Long term changes in the visual fields of patients with temporal lobe epilepsy using vigabatrin. *Br J Ophthalmol* **84:**788-790.

Herranz JL and Argumosa A (2000) Proposal for the treatment of children with West's syndrome. *Rev Neurol* **31:**578-583.

Hillman MA, Wilke RA, Yale SH, Vidaillet HJ, Caldwell MD, Glurich I, Berg RL, Schmelzer J and Burmester JK (2005) *Clin Med Res* **3:**137.

Hirschhorn JN, Lohmueller K, Byrne E and Hirschhorn K (2001) A comprehensive review of genetic association studies. *Genet Med* **4:**45-61.

Hisama FM, Mattson RH, Lee HH, Felice K and Petroff OA (2001) GABA and the ornithine delta-aminotransferase gene in vigabatrin-associated visual field defects. *Seizure* **10:**505-507.

Hosking SL and Hilton EJR (2002) Neurotoxic effects of GABA-transaminase inhibitors in the treatment of epilepsy: ocular perfusion and visual performance. *Ophthalmic Physiol Opt* **22:**440-447.

International HapMap Consortium (2005) A haplotype map of the human genome. *Nature* **437:**1299-1320.

Ioannidis JPA (2003) Genetic associations: False or true? *Trends Mol Med* **9:**135-138.

Ioannidis JPA, Ntzani EE, Trikalinos TA and Contopoulos-Ioannidis DG (2001) Replication validity of genetic association studies. *Nat Genet* **29:**306-309.

Izumi Y, Ishikawa M, Benz AM, Izumi M, Zorumski CF and Thio LL (2004) Acute vigabatrin retinotoxicity in albino rats depends on light but not GABA. *Epilepsia* **45:**1043-1048.

Jacob JN, Hesse GW and Shashoua VE (1990) Synthesis, brain uptake, and pharmacological properties of a glyceryl lipid containing GABA and the GABA-T inhibitor γ-vinyl-GABA. *J Med Chem* **33:**733-736.

James WL, Abelson MB, Ben-Menachem E, Pellock JM and Donald Shields W (2009) Vigabatrin: 2008 Update. *Epilepsia* **50:**163-173.

Jammoul F, Wang Q, Nabbout R, Coriat C, Duboc A, Simonutti M, Dubus E, Craft CM, Ye W and Collins SD (2009) Taurine deficiency is a cause of vigabatrin-induced retinal phototoxicity. *Ann Neurol* **65:**98-107.

Jeon SG, Bahn JH, Jang JS, Park J, Kwon O, Cho S and Choi SY (2000) Human brain GABA transaminase. *Eur J Biochem* **267:**5601-5607.

Joober R, Benkelfat C, Brisebois K, Toulouse A, Turecki G, Lal S, Bloom D, Labelle A, Lalonde P and Fortin D (1999) T102C polymorphism in the 5HT2A gene and schizophrenia: relation to phenotype and drug response variability. *J Psychiatry Neurosci* **24:**141.

Jorgensen AL and Williamson PR (2008) Methodological quality of pharmacogenetic studies: issues of concern. *Stat Med* **27:**6547-6569.

Jung MJ, Lippert B, Metcalf BW, Schechter PJ, Bohlen P and Sjoerdsma A (1977) The effect of 4-amino hex-5-ynoic acid (gamma-acetylenic GABA, gammma-ethynyl GABA) a catalytic inhibitor of GABA transaminase, on brain GABA metabolism in vivo. *J Neurochem* **28:**717-723.

Kaiser R, Könneker M, Henneken M, Dettling M, Müller-Oerlinghausen B, Roots I and Brockmöller J (2000) Dopamine D 4 receptor 48-bp repeat polymorphism: no association with response to antipsychotic treatment, but association with catatonic schizophrenia. *Mol Psychiatry* **5:**418-424.

Kaiser R, Sezer O, Papies A, Bauer S, Schelenz C, Tremblay P-, Possinger K, Roots I and Brockmölier J (2002) Patient-tailored antiemetic treatment with 5-hydroxytryptamine type 3 receptor antagonists according to cytochrome P-450 2D6 genotypes. *J Clin Oncol* **20:**2805-2811.

Kalow W (1962) Pharmacogenetics: heredity and the response to drugs, pp 231, W. B. Saunders Co., Philadelphia.

Kalow W and Staron N (1957) On distribution and inheritance of atypical forms of human serum cholinesterase, as indicated by dibucaine numbers. *Can J Med Sci* **35:**1305-1320.

Kim M-, Cho J-, Lim H-, Hong K-, Chung J-, Bae K-, Oh D-, Shin S-, Lee S-, Lee D-, Min B and Jang I- (2003) Effect of the CYP2D6 genotype on the pharmacokinetics of tropisetron in healthy Korean subjects. *Eur J Clin Pharmacol* **59:**111-116.

Kinirons P, Rabinowitz D, Gravel M, Long J, Winawer M, Sénéchal G, et al. (2008) Phenotypic concordance in 70 families with IGE-implications for genetic studies of epilepsy. *Epilepsy Res.* **82(1)**:21-28.

Kinirons P, Cavalleri GL, Singh R, Shahwan A, Acheson JF, Wood NW, Goldstein DB, Sisodiya SM, Doherty CP and Delanty N (2006a) A pharmacogenetic exploration of vigabatrin-induced visual field constriction. *Epilepsy Res* **70:**144-152.

Kinirons P, Cavalleri GL, O'Rourke D, Doherty CP, Reid I, Logan P, Liggan B and Delanty N (2006b) Vigabatrin Retinopathy in an Irish Cohort: Lack of Correlation with Dose. *Epilepsia* **47:**311-317.

Knapp J, Boknik P, Gumbinger HG, Linck B, Luss H, Muller FU, Schmitz W, Vahlensieck U and Neumann J (1999) Quantitation of clobazam in human

plasma using high-performance liquid chromatography. *J Chromatogr Sci* **37:**145-149.

Koeppen D (1985) A review of clobazam studies in epilepsy. in *Clobazam, Human Psychopharmacology and clinical applications* (I. Hindmarch, P. D. Stonier and M. R. Trimble eds) pp 1207-1215, Royal Society of Medicine, London.

Koeppen D, Baruzzi A, Capozza M, Chauvel P, Courjon J, Favel P, Harmant J, Lorenz H, Oller FV and Procaccianti G (1987) Clobazam in therapy-resistant patients with partial epilepsy: a double-blind placebo-controlled crossover study. *Epilepsia* **28:**495-506.

Krauss GL, Johnson MA and Miller NR (1998) Vigabatrin-associated retinal cone system dysfunction: Electroretinogram and ophthalmologic findings. *Neurology* **50:**614-618.

Kumlien E, Sherif F, Ge L and Oreland L (1995) Platelet and brain GABA-transaminase and monoamine oxidase activities in patients with complex partial seizures. *Epilepsy Res* **20:**161-170.

Kunicki PK (2001) Simple and sensitive high-performance liquid chromatographic method for the determination of 1, 5-benzodiazepine clobazam and its active metabolite N-desmethylclobazam in human serum and urine with application to 1, 4-benzodiazepines analysis. *J Chromatogr B Biomed Sci Appl* **750:**41-49.

Kwan P, Sills GJ and Brodie MJ (2001) The mechanisms of action of commonly used antiepileptic drugs. *Pharmacol Ther* **90:**21-34.

Larsson OM and Schousboe A (1990) Kinetic characterization of GABA-transaminase from cultured neurons and astrocytes. *Neurochem Res* **15:**1073-1077.

Lawden MC, Eke T, Degg C, Harding GFA and Wild JM (1999) Visual field defects associated with vigabatrin therapy. *BMJ* **67:**716.

Leach JP, Sills GJ, Butler E, Forrest G, Thompson GG and Brodie MJ (1997) Neurochemical actions of gabapentin in mouse brain. *Epilepsy Res* **27:**175-180.

Leach JP, Sills GJ, Majid A, Butler E, Carswell A, Thompson GG and Brodie MJ (1996) Effects of tiagabine and vigabatrin on GABA uptake into primary cultures of rat cortical astrocytes. *Seizure* **5:**229-234.

Lehmann H and Ryan E (1956) The familial incidence of low pseudocholinesterase level. *Lancet* **271:**124.

Lepper ER, Baker SD, Permenter M, Ries N, van Schaik RHN, Schenk PW, Price DK, Ahn D, Smith NF and Cusatis G (2005) Effect of common CYP3A4 and CYP3A5 variants on the pharmacokinetics of the cytochrome P450 3A phenotyping probe midazolam in cancer patients. *Clin Cancer Res* **11:**7398.

Levinson DF and Devinsky O (1999) Psychiatric adverse events during vigabatrin therapy. *Neurology* **53:**1503-1511.

Lewis P (1989) Introduction. *Br J Clin Pharmacol* **27:**1S.

Lingjaerde O (1969) Uptake of serotonin in blood platelets: Dependence on sodium and chloride, and inhibition by choline. *FEBS Lett* **3:**103-106.

Lippert B, Metcalf BW, Jung MJ and Casara P (1977) 4-Amino-hex-5-enoic Acid, a Selective Catalytic Inhibitor of 4-Aminobutyric-Acid Aminotransferase in Mammalian Brain. *Eur J Biochem* **74:**441-445.

Loiseau P, Hardenberg JP and Pestre M (1986) Double-blind, placebo-controlled study of vigabatrin (gamma-vinyl GABA) in drug-resistant epilepsy. *Epilepsia* **27:**115-120.

Loots GG, Locksley RM, Blankespoor CM, Wang ZE, Miller W, Rubin EM and Frazer KA (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288:**136.

Löscher W and Schmidt D (2006) Experimental and clinical evidence for loss of effect (tolerance) during prolonged treatment with antiepileptic drugs. *Epilepsia* **47:**1253-1284.

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350:**2129-2139.

Macdonald RL and Kelly KM (1995) Antiepileptic drug mechanisms of action. *Epilepsia* **36 Suppl 2:**S2-12.

Macdonald RL, Rogers CJ and Twyman RE (1989) Barbiturate regulation of kinetic properties of the GABAA receptor channel of mouse spinal neurones in culture. *J Physiol (Lond)* **417:**483-500.

Maitland ML, DiRienzo A and Ratain MJ (2006) Interpreting disparate responses to cancer therapy: The role of human population genetics. *J Clin Oncol* **24:**2151-2157.

Mangano RM and Schwarcz R (1982) Huntington's disease. Glutamate and aspartate metabolism in blood platelets. *J Neurol Sci* **53:**489-500.

Mangano RM and Schwarcz R (1981) The human platelet as a model for the glutamatergic neuron: platelet uptake of L-glutamate. *J Neurochem* **36:**1067-1076.

Manuchehri K, Goodman S, Siviter L and Nightingale S (2000) A controlled study of vigabatrin and visual abnormalities. *Br J Ophthalmol* **84:**499-505.

Masellis M, Basile V, Meltzer HY, Lieberman JA, Sevy S, Macciardi FM, Cola P, Howard A, Badri F and Nöthen MM (1998) Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients. *Neuropsychopharmacology* **19:**123-132.

McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JPA and Hirschhorn JN (2008) Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat Rev Genet* **9:**356-369.

McLeod HL (2002) Genetic strategies to individualize supportive care. *J Clin Oncol* **20:**2765-2767.

Meldrum BS (1996) Update on the mechanism of action of antiepileptic drugs. *Epilepsia* **37:**S4-11.

Meldrum BS and Chapman AG (1986) Benzodiazepine receptors and their relationship to the treatment of epilepsy. *Epilepsia* **27 Suppl 1:**S3-13.

Menachem EB, Persson LI, Schechter PJ, Haegele KD, Huebert N, Hardenberg J, Dahlgren L and Mumford JP (1988) Effects of single doses of vigabatrin on CSF concentrations of GABA, homocarnosine, homovanillic acid and 5-hydroxyindoleacetic acid in patients with complex partial epilepsy. *Epilepsy Res* **2:**96-101.

Meyer UA (2004) Pharmacogenetics - five decades of therapeutic lessons from genetic diversity. *Nat Rev Genet* **5:**669-676.

Michael B and Marson AG (2008) Clobazam as an add-on in the management of refractory epilepsy. *Cochrane Database Syst Rev* **(2):**CD004154.

Miller NR, Johnson MA, Paul SR, Girkin CA, Perry JD, Endres M and Krauss GL (1999) Visual dysfunction in patients receiving vigabatrin: clinical and electrophysiologic findings. *Neurology* **53:**2082-2087.

Mitchell WG and Shah NS (2002) Vigabatrin for infantile spasms. *Pediatr Neurol* **27:**161-164.

Motulsky AG (1957) Drug reactions enzymes, and biochemical genetics. *JAMA* **165:**835-837.

Munn R, Camfield P, Camfield C and Dooley J (1988) Clobazam for refractory childhood seizure disorders--a valuable supplementary drug. *Can J Neurol Sci* **15:**406-408.

Nebert DW (1999) Pharmacogenetics and pharmacogenomics: why is this relevant to the clinical geneticist? *Clin Genet* **56:**247-258.

Nebert DW and Vesell ES (2004) Advances in pharmacogenomics and individualized drug therapy:exciting challenges that lie ahead. *Eur J pharmacol* **500:**267-280.

Nebert DW, Zhang G and Vesell ES (2008) From Human Genetics and Genomics to Pharmacogenetics and Pharmacogenomics: Past Lessons, Future Directions. *Drug Metab.Rev.* 187-224.

Nebert DW and Vesell ES (2006) Can personalized drug therapy be achieved? A closer look at pharmaco-metabonomics. *Trends Pharmacol Sci* **27:**580-586.

Newman WD, Tocher K and Acheson JF (2002) Vigabatrin associated visual field loss: a clinical audit to study prevalence, drug history and effects of drug withdrawal. *Eye* **16:**567-571.

Niederhauser S and Mojon DS (2000) Normal isopter position in the peripheral visual field in Goldmann kinetic perimetry. *Ophthalmologica* **216:**406-408.

Nieminen T, Kähönen M, Viiri LE, Grönroos P and Lehtimäki T (2008) Pharmacogenetics of apolipoprotein E gene during lipid-lowering therapy: Lipid levels and prevention of coronary heart disease. *Pharmacogenomics* **9:**1475-1486.

Nousiainen I, Kälviäinen R and Mäntyjärvi M (2000a) Color vision in epilepsy patients treated with vigabatrin or carbamazepine monotherapy. *Ophthalmology* **107:**884-888.

Nousiainen I, Kälviäinen R and Mäntyjärvi M (2000b) Contrast and glare sensitivity in epilepsy patients treated with vigabatrin or carbamazepine monotherapy compared with healthy volunteers. *Br J Ophthalmol* **84:**622-625.

Oset-Gasque MJ, Launay JM and Gonzalez MP (1986) GABAergic mechanisms in blood cells: their possible role. in *GABAergic Mechanisms in the Mamm lian Periphery* (S. L. Erdo and N. G. Bowery eds) pp 305-324, Raven Press, New York.

Ovation Pharmaceuticals I (2007) Vigabatrin induced retinal dysfunction. *Data on File*

Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M and Nakamura Y (2002) Functional SNPs in the lymphotoxin-α gene that are associated with susceptibility to myocardial infarction. *Nat Genet* **32:**650-654.

Paasonen MK (1968) Platelet 5-hydroxytryptamine as a model in pharmacology. *Ann Med Exp Biol Fenn* **46:**416-422.

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304:**1497-1500.

Patsalos PN (2005) Properties of Antiepileptic Drugs in the Treatment of Idiopathic Generalized Epilepsies. *Epilepsia* **46:**140-148.

Peedicayil J (2008) Pharmacoepigenetics and pharmacoepigenomics. *Pharmacogenomics* **9:**1785-1786.

Pirmohamed M and Park BK (2001) Genetic susceptibility to adverse drug reactions. *Trends Pharmacol Sci* **22:**298-305.

Pistos C and Stewart JT (2003) Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column. *J Pharm Biomed Anal* **33:**1135-1142.

Pitts FN,Jr, Quick C and Robins E (1965) The Enzymic Measurement of Gamma-Aminobutyric-Alpha-Oxoglutaric Transaminase. *J Neurochem* **12:**93-101.

Poolsup, Po LW and Knight (2000) Pharmacogenetics and psychopharmacotherapy. *J Clin Pharm Ther* **25:**197-220.

Proença P, Teixeira H, Pinheiro J, Marques EP and Vieira DN (2004) Forensic intoxication with clobazam: HPLC/DAD/MSD analysis. *Forensic Sci Int* **143:**205-209.

Rainesalo S, Saransaari P, Peltola J and Keränen T (2003) Uptake of GABA and activity of GABA-transaminase in platelets from epileptic patients. *Epilepsy Res* **53:**233-239.

Ratnaraj N, Goldberg V and Lascelles PT (1984) Determination of clobazam and desmethylclobazam in serum using high-performance liquid chromatography. *Analyst* **109:**813-815.

Remy C (2007) Clobazam in the treatment of epilepsy: a review of the literature. *Epilepsia* **35:**S88-S91.

Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, Blough DK, Thummel KE, Veenstra DL and Rettie AE (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* **352:**2285-2293.

Rigaud AS, Traykov L, Caputo L, Guelfi MC, Latour F, Couderc R, Moulin F, de Rotrou J, Forette F and Boller F (2000) The apolipoprotein E epsilon4 allele and the response to tacrine therapy in Alzheimer's disease. *Eur J Neurol* **7:**255-258.

Rimmer E, Kongola G and Richens A (1988) Inhibition of the enzyme, GABA-aminotransferase in human platelets by vigabatrin, a potential antiepileptic drug. *Br J Clin Pharmacol* **25:**251-259.

Risch N and Merikangas K (1996) The future of genetic studies of complex human diseases. *Science* **273:**1516-1517.

Riva A and Kohane IS (2004) A SNP-centric database for the investigation of the human genome. *BMC Bioinformatics* **5:**33.

Rodgers GM (1999) Overview of platelet physiology and laboratory evaluation of platelet function. *Clin Obstet Gynecol* **42:**349-359.

Ross DF, Fishman GA, Gilbert LD and Anderson RJ (1984) Variability of visual field measurements in normal subjects and patients with retinitis pigmentosa. *Arch Ophthalmol* **102:**1004.

Rothman DL, Petroff OA, Behar KL and Mattson RH (1993) Localized 1H NMR measurements of gamma-aminobutyric acid in human brain in vivo. *Proc Natl Acad Sci U S A* **90:**5662-5666.

Rouini M, Ardakani YH, Hakemi L, Mokhberi M and Badri G (2005) Simultaneous determination of clobazam and its major metabolite in human plasma by a rapid HPLC method. Chromatogr B Analyt Technol Biomed Life Sci**823:**167-171.

Rowley HL, Marsden CA and Martin KF (1995) Differential effects of phenytoin and sodium valproate on seizure-induced changes in gamma-aminobutyric acid and glutamate release in vivo. *Eur J Pharmacol* **294:**541-546.

Rupp W, Badian M, Christ O, Hajdu P, Kulkarni RD, Taeuber K, Uihlein M, Bender R and Vanderbeke O (1979) Pharmacokinetics of single and multiple doses of clobazam in humans. *Br J Clin Pharmacol* **7 Suppl 1:**51S-57S.

Satishchandra P, Kashyap B and Goverdhan S (1999) Vigabatrin in intractable complex partial epilepsy. *Epilepsia* **40:**256.

Schachter SC (1995) Review of the mechanisms of action of antiepileptic drugs. *CNS Drugs* **4:**469-477.

Schechter PJ, Hanke NF, Grove J, Huebert N and Sjoerdsma A (1984) Biochemical and clinical effects of gamma-vinyl GABA in patients with epilepsy. *Neurology* **34:**182-186.

Scholten AH, Welling PL, Brinkman UA and Frei RW (1980) Use of PTFE coils in post-column photochemical reactors for liquid chromatography--application to pharmaceuticals. *J Chromatogr* **199:**239-248.

Schousboe A, Wu JY and Roberts E (1973) Purification and characterization of the 4-aminobutyrate-2-ketoglutarate transaminase from mouse brain. *Biochemistry (N Y)* **12:**2868-2873.

Sconce EA, Avery PJ, Wynne HA and Kamali F (2008) Vitamin K epoxide reductase complex subunit 1 (VKORC1) polymorphism influences the anticoagulation response subsequent to vitamin K intake: A pilot study. *J Thromb Haemost* **6:**1226-1228.

Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata K, Kawashima S and Tawa R (2001) Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res* **103:**399-409.

Seo T, Nagata R, Ishitsu T, Murata T, Takaishi C, Hori M and Nakagawa K (2008) Impact of CYP2C19 polymorphisms on efficacy of clobazam therapy. *Pharmacogenomics* **9:**527-537.

Sherif F, Gottfries CG, Alafuzoff I and Oreland L (1992a) Brain gamma-aminobutyrate aminotransferase (GABA-T) and monoamine oxidase (MAO) in patients with Alzheimer's disease. *J Neural Transm Park Dis Dement Sect* **4:**227-240.

Sherif F, Hallman J and Oreland L (1992b) Low platelet gamma-aminobutyrate aminotransferase and monoamine oxidase activities in chronic alcoholic patients. *Alcohol Clin Exp Res* **16:**1014-1020.

Sherif F, Eriksson L and Oreland L (1991) GABA-transaminase activity in rat and human brain: regional, age and sex-related differences. *J Neural Transm Gen Sect* **84:**95-102.

Sherif FM (1994) GABA-transaminase in brain and blood platelets: basic and clinical aspects. *Prog Neuropsychopharmacol Biol Psychiatry* **18:**1219-1233.

Sherif FM and Ahmed SS (1995) Basic aspects of GABA-transaminase in neuropsychiatric disorders. *Clin Biochem* **28:**145-154.

Sherif FM, Jossan SS and Oreland L (1993) gamma-Aminobutyrate aminotransferase activity in blood platelets of six species. *Comp Biochem Physiol C* **104:**345-349.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270:**414.

Shinnar S (2010) The new ILAE classification. *Epilepsia* **51:**715-717.

Shorvon SD (1998) The use of clobazam, midazolam, and nitrazepam in epilepsy. *Epilepsia* **39:**S15-S23.

Shorvon SD (1995) Clobazam, in *Antiepileptic drugs* (R. H. Levy, R. H. Mattson and B. Meldrum eds) pp 763-778, Ravenn Press, New York.

Sieghart W (1992) GABAA receptors: ligand-gated Cl- ion channels modulated by multiple drug-binding sites. *Trends Pharmacol Sci* **13:**446-450.

Sills GJ, Leach JP, Butler E, Carswell A, Thompson GG and Brodie MJ (1996) Antiepileptic drug action in primary cultures of rat cortical astrocytes. *Epilepsia* **37 (suppl. 4):**116.

Sills GJ, Patsalos PN, Butler E, Forrest G, Ratnaraj N and Brodie MJ (2001) Visual field constriction: Accumulation of vigabatrin but not tiagabine in the retina. *Neurology* **57:**196-200.

Sim SC, Risinger C, Dahl M, Aklillu E, Christensen M, Bertilsson L and Ingelman-Sundberg M (2006) A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants[ast]. *Clin Pharmacol Ther* **79:**103-113.

Snyder LH (1932) Studies in Human Inheritance. IX, The Inheritance of Taste Deficiency in Man. *Ohio J Sci* **32:**436-468.

Stewart JT, Honigberg IL, Tsai AY and Hajdu P (1979) Fluorometric determination of clobazam, a 1,5-benzodiazepine, in human plasma. *J Pharm Sci* **68:**494-496.

Storici P, Capitani G, De Biase D, Moser M, John RA, Jansonius JN and Schirmer T (1999) Crystal structure of GABA-aminotransferase, a target for antiepileptic drug therapy. *Biochemistry* **38:**8628-8634.

Streete JM, Berry DJ and Newbery JE (1991) The analysis of clobazam and its metabolite desmethylclobazam by high-performance liquid chromatography. *Ther Drug Monit* **13:**339.

Swen JJ, Huizinga TW, Gelderblom H, De Vries EGE, Assendelft WJJ, Kirchheiner J and Guchelaar H- (2007) Translating pharmacogenomics: Challenges on the road to the clinic. *PLoS Medicine* **4:**1317-1324.

Tollman P, Guy P, Altshuler J, Flanagan A and Steiner M (2001) A revolution in R&D: How genomics and genetics are transforming the biopharmaceutical industry. *The Boston Consulting Group*

Tomasini JL, Bun H, Coassolo P, Aubert C and Cano JP (1985) Determination of clobazam, N-desmethylclobazam and their hydroxy metabolites in plasma and urine by high-performance liquid chromatography. *J Chromatogr* **343:**369-377.

Tsuji M, Aida N, Obata T, Tomiyasu M, Furuya N, Kurosawa K, Errami A, Gibson KM, Salomons GS and Jakobs C (2010) A new case of GABA transaminase deficiency facilitated by proton MR spectroscopy. *J Inherit Metab Dis* **33:**85-90.

Twyman RE, Rogers CJ and Macdonald RL (1989) Differential regulation of gamma-aminobutyric acid receptor channels by diazepam and phenobarbital. *Ann Neurol* **25:**213-220.

Upton N (1994) Mechanisms of action of new antiepileptic drugs: rational design and serendipitous findings. *Trends Pharmacol Sci* **15:**456-462.

Vatsis KP, Weber WW, Bell DA, Dupret JM, Evans DA, Grant DM, Hein DW, Lin HJ, Meyer UA and Relling MV (1995) Nomenclature for N-acetyltransferases. *Pharmacogenetics* **5:**1-17.

Vogel F (1959) II. Moderne Probleme der Humangenetik. *Ergeb Inn Med Kinderheild* **12:**52-125.

Voora D, McLeod HL, Eby C and Gage BF (2005) The pharmacogenetics of coumarin therapy. *Pharmacogenomics* **6:**503-513.

Wadelius M, Chen LY, Downes K, Ghori J, Hunt S, Eriksson N, Wallerman O, Melhus H, Wadelius C, Bentley D and Deloukas P (2005) Common VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics* **5:**262-270.

Walsh T, Casadei S, Coats KH, Swisher E, Stray SM, Higgins J, Roach KC, Mandell J, Lee MK, Ciernikova S, Foretova L, Soucek P and King M- (2006) Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *JAMA* **295:**1379-1388.

Wang J, Hug D, Gautschi K and Wieser HG (1993) Clobazam for treatment of epilepsy. *J Epilepsy* **6:**180-184.

Weber WW (2001) Effect of pharmacogenetics on medicine. *Environ Mol Mutagen* **37:**179-184.

Weber KH, Bauer A and Hauptmann KH (1972) Benzodiazepines with psychotropic activity. 3. N-aryl- and N-heteroaryl-1H-1,5-benzodiazepine-2,4-(3H,5H)-diones. *Justus Liebigs Ann Chem* **756:**128-138.

Wheless JW, Ramsay RE and Collins SD (2007) Vigabatrin. *Neurotherapeutics* **4:**163-172.

White HL and Sato TL (1978) GABA-transaminases of human brain and peripheral tissues-kinetic and molecular properties. *J Neurochem* **31:**41-47.

White HL (1979) 4-Aminobutyrate:2-Oxoglutarate Aminotransferase in Blood Platelets. *Science* **205:**696-698.

White HS (1999) Comparative anticonvulsant and mechanistic profile of the established and newer antiepileptic drugs. *Epilepsia* **40 Suppl 5:**S2-10.

White HL and Faison LD (1980) GABA-T in blood platelets: Comparison with GABA-T of other tissues. *Brain Res Bull* **5:**115-119.

Wieling J (2002) LC-MS-MS experiences with internal standards. *Chromatographia* **55:**107-113.

Wild JM, Martinez C, Reinshagen G and Harding CF (1999) Characteristics of a Unique Visual-Field Defect Attributed to Vigabatrin. *Epilepsia* **40:**1784-1794.

Wild JM, Ahn HS, Baulac M, Bursztyn J, Chiron C, Gandolfo E, Safran AB, Schiefer U and Perucca E (2007) Vigabatrin and epilepsy: lessons learned. *Epilepsia* **48:**1318-1327.

Williams RJ (1956) *Biochemical Individuality.* John Wiley & Sons, New York,USA.

Zieve PD and Solomon HM (1968) Uptake of amino acids by the human platelet. *Am J Physiol* **214:**58-61.

Zilli MA and Nisi G (1986) Simple and sensitive method for the determination of clobazam, clonazepam and nitrazepam in human serum by high-performance liquid chromatography. *J Chromatogr* **378:**492-497.

Appendix 1. Classification of epilepsy according to the Commission on Classification and Terminology of the International League Against Epilepsy, 2010.

Table 1. Classification of seizures [a]

Generalized seizures
Tonic–clonic (in any combination)
Absence
Typical
Atypical
Absence with special features
Myoclonic absence
Eyelid myoclonia
Myoclonic
Myoclonic
Myoclonic atonic
Myoclonic tonic
Clonic
Tonic
Atonic
Focal seizures
Unknown
Epileptic spasms

[a]Seizure that cannot be clearly diagnosed into one of the preceding categories should be considered unclassified until further information allows their accurate diagnosis. This is not considered a classification category, however.

Table 2. Descriptors of focal seizures according to degree of impairment during seizure [a]

Without impairment of consciousness or awareness

With observable motor or autonomic components. This roughly corresponds to the concept of ''simple partial seizure. ''Focal motor'' and ''autonomic'' are terms that may adequately convey this concept depending on the seizure manifestations).

Involving subjective sensory or psychic phenomena only.

This corresponds to the concept of an aura, a term endorsed in the 2001 Glossary. With impairment of consciousness or awareness. This roughly corresponds to the concept of complex partial seizure. ''Dyscognitive'' is a term that has been proposed for this concept (Blume et al., 2001).

Evolving to a bilateral, convulsive[b] seizure

(Involving tonic, clonic, or tonic and clonic components). This expression replaces the term ''secondarily generalized seizure.''

[a]For more descriptors that have been clearly defined and recommended for use, please see Blume et al., 2001.

[b]The term ''convulsive'' was considered a lay term in the Glossary; however, we note that it is used throughout medicine in various forms and translates well across many languages. Its use is, therefore, endorsed.

Table 3. Electroclinical syndromes and other epilepsies

Electroclinical syndromes arranged by age at onset[a]

Neonatal period

- Benign familial neonatal epilepsy (BFNE)
- Early myoclonic encephalopathy (EME)
- Ohtahara syndrome

Infancy

- Epilepsy of infancy with migrating focal seizures
- West syndrome
- Myoclonic epilepsy in infancy (MEI)
- Benign infantile epilepsy
- Benign familial infantile epilepsy
- Dravet syndrome
- Myoclonic encephalopathy in nonprogressive disorders

Childhood

- Febrile seizures plus (FS+) (can start in infancy)
- Panayiotopoulos syndrome
- Epilepsy with myoclonic atonic (previously astatic) seizures
- Benign epilepsy with centrotemporal spikes (BECTS)
- Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)
- Late onset childhood occipital epilepsy (Gastaut type)
- Epilepsy with myoclonic absences
- Lennox-Gastaut syndrome
- Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS)[b]
- Landau-Kleffner syndrome (LKS)
- Childhood absence epilepsy (CAE)

Adolescence – Adult

- Juvenile absence epilepsy (JAE)
- Juvenile myoclonic epilepsy (JME)
- Epilepsy with generalized tonic–clonic seizures alone
- Progressive myoclonus epilepsies (PME)
- Autosomal dominant epilepsy with auditory features (ADEAF)
- Other familial temporal lobe epilepsies

Less specific age relationship

- Familial focal epilepsy with variable foci (childhood to adult)
- Reflex epilepsies

Distinctive constellations

- Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS)
- Rasmussen syndrome
- Gelastic seizures with hypothalamic hamartoma
- Hemiconvulsion–hemiplegia–epilepsy

Epilepsies that do not fit into any of these diagnostic categories can be distinguished first on the basis of the presence or absence of a known structural or metabolic condition (presumed cause) and then on the basis of the primary mode of seizure onset (generalized vs. focal).

Epilepsies attributed to and organized by structural-metabolic causes

Malformations of cortical development (hemimegalencephaly, heterotopias, etc.)
Neurocutaneous syndromes (tuberous sclerosis complex, Sturge-Weber, etc.)
Tumor
Infection
Trauma

Angioma

Perinatal insults
Stroke
Etc.

Epilepsies of unknown cause

Conditions with epileptic seizures that are traditionally not diagnosed as a form of epilepsy per se
Benign neonatal seizures (BNS)
Febrile seizures (FS)

[a]The arrangement of electroclinical syndromes does not reflect etiology.
[b]Sometime referred to as Electrical Status Epilepticus during Slow Sleep(ESES).